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(54) Title: RECOMBINANT HUMAN METAPNEUMOVIRUS AND ITS USE

(57) Abstract: Recombinant HMPV (rHMPV) and related immunogenic compositions and methods are provided. The rHMPVs, including chimeric and HMPV vector viruses, provided according to the current disclosure are infectious and attenuated in permissive mammalian subjects, including humans. The rHMPVs are useful in immunogenic compositions for eliciting an immune response against HPIV, against one or more non-HMPV pathogens, or against a HMPV and a non-HMPV pathogen. Also provided are isolated polynucloetide molecules and vectors incorporating a recombinant HMPV genome or antigenome.

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RECOMBINANT HUMAN METAPNEUMOVIRUS AND ITS USE

PRIORITY CLAIM

This application claims the benefit of U.S. Provisional Patent Application No. 60/451,119,
filed February 28, 2003, and U.S. Provisional Patent Application No. 60/478,667, filed June 13,
2003. Both of the provisional applications are incorporated by reference herein in their entirety.

FIELD OF THE DISCLOSURE

This invention relates to the field of virology and, more specifically, to methods for
producing recombinat human metapneumovirus (rHMPV), for producing replication competent
derivatives with desirable properties such as attenuation and protective efficacy, and to uses of
rHMPV in immunoprophylaxis and therapy.

BACKGROUND

Human metapneumovirus (HMPV) is a virus that was first recovered in the Netherlands
from infants and children experiencing acute respiratory tract disease (van den Hoogen *et al.* Nat.
Med. 7:719-724, 2001; De Jong *et al.* WO 02/057/302 A2).

HMPV is worldwide in prevalence and resembles human respiratory syncytial virus (RSV or
HRSV) with regard to disease signs and the ability to infect and cause disease in the young infant as
well as individuals of all ages (for a review, see Heikkinen and Jarvinen, Lancet 361:51-59, 2003).

HMPV is characterized as an enveloped virus with a genome that is a single negative strand
of RNA of approximately 13 kb. The virus has been classified presumptively in the
Metapneumovirus genus, Pneumovirus subfamily, Paramyxovirus family of the Order
Mononegavirales, comprising the nonsegmented negative strand RNA viruses or mononegaviruses.
Mononegaviruses also are called nonsegmented negative strand RNA viruses. The Paramyxovirus
family has two subfamilies, Paramyxovirinae and Pneumovirinae (also referred to as
paramyxoviruses and pneumoviruses, respectively).

Several other mononegaviruses are important agents of respiratory tract disease in pediatric
and other populations, for example, RSV and human parainfluenza virus types 1, 2 and 3 (HPIV1,
HPIV2 and HPIV3). Although these viruses have some similarities with HMPV, their sequences are
different from the HMPV viruses disclosed herein.

Since HMPV was described only recently, there is little documented experience with its
propagation, manipulation and stability, and there are no well established or widely available
reference strains, mutant strains, reported vaccine candidates, or reference virus-specific antibodies or
comparable reagents or systems or experimental animals models to facilitate characterization.
Because HMPV is associated with severe respiratory tract disease, there is a need to develop methods

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to engineer safe and effective vaccines to alleviate the serious health problems attributable to this
pathogen, particularly among young infants.

BRIEF SUMMARY OF SPECIFIC EMBODIMENTS

5   HMPV is a significant agent of human respiratory tract disease. Methods and compositions
are provided herein for recovering infectious, recombinant HMPV. Recombinant HMPV is disclosed
herein, including the complete nucleic acid sequence encoding all of the protein products of HMPV.
Compositions and methods for introducing defined, predetermined structural and phenotypic changes
into an infectious HMPV are disclosed, as are attenuated forms of HMPV as well as forms of HMPV
10   that have been modified to have improved qualities relevant to immunogenicity, safety, protective
efficacy, and breadth of vaccine coverage. Thus, methods are disclosed for generating an immune
response against HMPV in a subject. The methods include administering to the subject an attenuated
HMPV, thereby producing the immune response. Also described is the identification of a set of viral
ORFs whose expression is sufficient to direct viral transcription and RNA replication, and to produce
15   infectious HMPV entirely from cDNA.

The foregoing and other features and advantages will become more apparent from the
following detailed description of several embodiments, which proceeds with reference to the
accompanying figures.

20   **BRIEF DESCRIPTION OF THE FIGURES**

FIG. 1 is a diagrammatic representation of the negative sense genomes of HMPV strain
CAN97-83 (hereafter referred to as 83 or CAN-83) and RSV strain A2, showing a comparison of the
two genomes. Individual proposed genes are shown as shaded boxes separated by proposed
25   intergenic regions represented by open bars. The 3′ extragenic leader and 5′ extragenic trailer regions
also are shown as open boxes. Tentative HMPV gene assignments are based on ORFs whose
potential protein exhibits partial amino acid sequence identity or structural similarity with a known
protein from RSV. Note that HMPV lacks apparent counterparts to the NS1 and NS2 genes present
in RSV (boxed), and that the putative F and M2 gene pair precedes the SH-G gene pair in HMPV but
30   follows it in RSV (the difference in the position of SH-G is indicated with dotted lines). The M2 and
L genes overlap in RSV but may not do so in HMPV. In both viruses, the M2 gene contains two
ORFs M2-1 and M2-2, depicted as filled bars. Amino acid lengths are shown for the deduced
unmodified viral proteins. Each of the RSV proteins has been directly identified; none of the
proposed HMPV proteins had been directly identified prior to the current disclosure. Abbreviations
35   (with HMPV assignments by analogy to RSV): N, major nucleocapsid protein; P, nucleocapsid
phosphoprotein; M, inner virion matrix protein; M2-1 and M2-2, products of the first and second
ORFs, respectively, in the M2 mRNA; SH, small hydrophobic protein; G, heavily glycosylated

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protein involved in attachment; F, fusion protein involved in penetration; L, large polymerase protein; NS1 and NS2, nonstructural protein 1 and 2, respectively. This and all subsequent diagrams are not to scale.

5   FIG. 2 is a diagram showing sequence alignments between the 3′ leader (top) and 5′ trailer (bottom) regions and flanking areas of the genome of HMPV strain 83 (SEQ ID NO: 18 and SEQ ID NO: 22, respectively), avian pneumovirus (APV or AMPV, SEQ ID NO: 19 and SEQ ID NO: 23, respectively), and HRSV strain A2 (SEQ ID NO: 20 and SEQ ID NO: 24, respectively). The sequences are in genome (negative) sense. Nucleotide assignments that are the same in two or more sequences between HMPV 83, APV and RSV are shaded; sequence gaps introduced to make optimal

10 alignments are indicated by dashes. The bars over the left hand end of each sequence indicates regions whose spacing and sequence had not been previously determined for any HMPV. The partial sequences available for HMPV strain 00-1 3′ leader and 5′ trailer regions (SEQ ID NO: 17 and SEQ ID NO: 21, respectively) are indicated at the top: nucleotide assignments in strain 00-1 that are identical to those of HMPV 83 are indicated by asterisks while nucleotide differences are indicated.

15 Sequences: APV (Randhawa et al., J. Virol. 71:9849-54, 1997), RSV (Mink et al., Virology 185:615-24, 1991), HMPV 00-1 (van den Hoogen et al., Nat. Med. 7:719-24, 2001).

        FIG. 3 is a diagram showing sequence alignments between the 3′ and 5′ ends of the genome of HMPV strain 83 (top; bases 1-41 of SEQ ID NO: 18 and bases 1-40 of SEQ ID NO: 22, respectively) and APV (bottom; bases 1-41 of SEQ ID NO: 19 and bases 1-42 of SEQ ID NO: 23,

20 respectively), with complementary nucleotides boxed. This shows that the last 13 nucleotides of the two ends of APV are perfect complements, whereas HMPV unexpectedly has two noncomplementary nucleotides. Sequences for HMPV that were not previously available are indicated with solid lines above (leader, Le strand) or below (trailer, Tr strand) the sequence.

        FIG. 4 is a diagram showing several examples of differences in nucleotide and amino acid

25 lengths between HMPV strains 83 and 00-1. The differences in G are illustrated by partial amino acid sequences (upper alignment; amino acids 190-219 of SEQ ID NO: 6 and amino acids 190-236 of SEQ ID NO: 11, respectively) and partial nucleotide sequences (second alignment; bases 6871-6929 of SEQ ID NO: 1 and bases 6886-6964 of SEQ ID NO: 36, respectively). The sequences are numbered according to the amino acid sequence of HMPV 83 G (top alignment) or the complete

30 nucleotide sequence of the antigenome of HMPV 83 (second alignment). Examples of differences include, (1) the SH protein for 83 is shorter by 4 aa due to an earlier stop codon; (2) the G protein for 83 is shorter by 17 aa due to an earlier stop codon, which is followed by a deletion in the downstream noncoding region; and (3) the intergenic region between the F and M2 genes of 83 is 13 nucleotides (nt) compared to 41 nt for 00-1. Taken together, these show that the G ORF and encoded G protein

35 of strain 83 are shorter by the equivalent of 17 amino acids from that of strain 00-1.

        FIG. 5 is a chart showing the percent amino acid sequence identity for the predicted, putative proteins of HMPV 83 compared to those of two other strains of HMPV (00-1, and 97-82),

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three different antigenic types of APV (A, B and C), the two subgroups of human RSV (A and B), bovine RSV (BRSV), and pneumonia virus of mice (PVM, a murine counterpart of HRSV). Note that HMPV is most closely related to APV C. ND indicates that a comparison was not done for the indicated subject.

5   FIG. 6 is a taxonomy tree, illustrating putative phylogenetic relationships between HMPV and other paramyxoviruses based on comparisons of the amino acid sequences of proposed matrix M proteins. Pneumovirinae and Paramyxovirinae are the two subfamilies of the Paramyxovirus family. The other taxonomic names refer to genera. The numbers refer to the extent of amino acid sequence divergence (percentage non-identity) divided by 100; hence, 0.2 refers to 20% amino acid

10   differences. Note that a system for recovering and modifying infectious recombinant virus has not been reported for any member of genus Metapneumovirus even though the avian members of this genus have been known for almost 25 years.

FIG. 7 is a diagram illustrating the construction of a cDNA encoding the complete antigenome of HMPV strain 83, designed from the complete consensus genomic sequence. Three

15   separate subgenomic cloned fragments were created: fragment 1 contains the putative N, P and M genes and is bordered on the upstream (left) side by an added promoter for bacteriophage T7 RNA polymerase (T7p) and on the right hand side by an *Nhe*I site that was added to the putative M-F intergenic region as a marker. The sequence changes involved in introducing the *Nhe*I site are shown. The T7 promoter was designed to add three nonviral G residues to the 5′ end of the antigenome, a

20   configuration chosen to improve the efficiency of the T7 promoter. Fragment 2 included the putative F, M2, SH and G genes and is bordered on the upstream side by the added *Nhe*I site and on the downstream side by a naturally occurring *Acc*65I site. Fragment 3 consists of the L gene followed by part of the hepatitis delta virus ribozyme (HDVribo) (Perrotta and Been, Nature 350:434-6, 1991) bordered by an *Rsr*II site that occurs naturally within that ribozyme. The vector for cloning and

25   expressing the HMPV 83 antigenome cDNA was pBSKSII, which is a derivative of a modified Bluescript II KS + (Stratagene) vector described previously (Durbin *et al.*, Virology 71:4272-7, 1997) and contains the hepatitis delta virus ribozyme followed by a terminator for T7 RNA polymerase (T7t). This vector was modified by the insertion of a polylinker containing *Ava*I, *Aat*II, *Nhe*I, and *Acc*65I sites, which in turn served to accept the cloned fragments 1, 2 and 3. The final cDNA encodes

30   the complete 13335-nucleotide HMPV 83 antigenome containing three added nonviral G residues at the 5′ end. The complete pHMPV-83 recombinant plasmid ("antigenome plasmid") contains 16333 bp.

FIG. 8 is a diagram showing putative transcription signals for HMPV strain 83 based on the identification of semi-conserved sequence motifs located between the major ORFs in the complete

35   sequence. Sequences are in positive (mRNA) sense. The semi-conserved sequence motif that precedes most of the ORFs in the complete sequence is shown as the putative gene start (GS) signal, with some of the most highly conserved sequences shown in upper case letters and with flanking

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sequence on either side in lower case. The individual GS motifs are named according to the putative
ORF that each precedes (SEQ ID NO: 1, bases 29-64 for N; bases 1237-1272 for P; bases 2154-2189
for M; bases 3041-3076 for F; bases 4698-4733 for M2; bases 5455-5490 for SH; bases 6206-6241
for G; and bases 7107-7142 for L). A consensus sequence is shown underneath (SEQ ID NO: 25).

5   For positions where alternative assignments can occur, these are listed below the consensus (SEQ ID
NOs: 26 and 27). By this analysis, the major element of the GS signal is contained within sixteen
nucleotides, of which positions 1, 3, 4, 6, 7, 9, and 14-16 were exactly conserved, with 14-16 also
serving as potential translation start sites. Similarly, the semi-conserved sequence motif that follows
most of the ORFs in the complete sequence is shown as the putative gene end (GE) signal, with some

10   of the most highly conserved sequences shown in upper case and flanking sequence in lower case.
The individual GE motifs are named according to the ORF that each follows (SEQ ID NO: 1, bases
1230-1249 for N; bases 2141-2166 for P; bases 3002-3032 for M; bases 4680-4710 for F; bases
5440-5467 for M2; bases 6077-6107 for SH; bases 6912-6942 for G; and bases 13223-13253 for L).
A consensus sequence is shown underneath (SEQ ID NO: 28). For positions where alternative

15   assignments can occur, these are listed below the consensus (SEQ ID NOs: 29 and 30). By this
analysis, the major element of the GE signal is contained within 12-13 nucleotides, of which
positions 1-3 and 10-12 are exactly conserved.

FIG. 9 is a diagram illustrating the construction of a transcription cassette containing the
ORF for jellyfish GFP under the control of HMPV transcription GS and GE signals, and insertion of

20   this cassette into the HMPV strain 83 antigenomic cDNA clone following antigenomic position 41.
Sequence representing part of the 3′ leader region (bases 23-41 of SEQ ID NO: 1) and the putative N
GS signal and beginning of the putative N ORF (bases 42-66 of SEQ ID NO: 1) is shown at the
bottom. The GFP transcription cassette consists of a 714-nucleotide cDNA containing the GFP ORF
flanked on the upstream side by 16 nucleotides (which include the initiating ATG of the GFP ORF)

25   representing the conserved putative N GS signal (bases 42-57 of SEQ ID NO: 1) and flanked on the
downstream side by 13 nucleotides representing the putative F GE signal (bases 4685-4697 of SEQ
ID NO: 1; which include the termination codon of the GFP ORF) followed by the trinucleotide AGT
designed to function as an intergenic (IG) region. The total length of the transcription cassette
including the trinucleotide intergenic region is 748 nucleotides. The F GE signal contains a naturally

30   occurring PacI site (italicized).

FIG. 10 is a diagram illustrating the construction of expression vectors comprising support
plasmids that contain ORFs corresponding to the proposed nucleocapsid and polymerase proteins of
HMPV strain 83: N, P, M2-1 and L. These ORFs were clarified in part based on sequence relatedness
to the respective RSV ORF (see, for example, Figure 5). Each ORF was placed under the control of a

35   T7 transcription promoter (T7p) and terminator (T7t). The L cDNA was assembled from two
overlapping pieces. Each ORF conformed to the consensus sequence of HMPV 83.

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FIG. 11 is a diagram showing the strategy for recovery of a recombinant version of HMPV strain 83, in this case expressing GFP as an added marker gene. Plasmids encoding the HMPV-GFP antigenome and the N, P, L and M2-1 support plasmids are transfected into BSR T7/5 cells, which are a derivative of the baby hamster kidney-21 (BHK-21) cell line that was engineered to

5   constitutively express T7 RNA polymerase (Buchholz *et al.*, J. Virol. 73:251-259. 1999). The support plasmids were constructed to encode the proposed HMPV nucleocapsid and polymerase proteins. By this strategy, the expression of the antigenome together with HMPV nucleocapsid/polymerase proteins provides the components for the self-assembly of HMPV nucleocapsids functional for transcription and RNA replication. These nucleocapsids then express all of the HMPV RNAs and

10   proteins, leading to a productive infection that produces rHMPV.

FIG. 12A is a digitized image of an agarose gel showing that the recovered recombinant HMPV-GFP (strain 83) contains the *Nhe*I site that was engineered into the antigenome cDNA and is not present in biologically-derived strain 83 parent. LLC-MK2 cells in T25 flask were infected with an aliquot of the supernatant from the transfected BSR T7/5 cells (first passage) and incubated at

15   32°C in the presence of 5 μg/ml trypsin. Eleven days post-infection, 1 ml of the supernatant was harvested, clarified by centrifugation at 1500 rpm for 5 minutes and used to extract the viral genomic RNA with the QIAamp viral RNA purification kit (QIAGEN) according to the manufacturer's recommendations. The RNA was subjected to reverse transcription (RT) using a positive sense primer designed to hybridize within the M gene (nucleotides 2719 to 2738 in the HMPV antigenome

20   exclusive of GFP). Polymerase chain reaction (PCR) was performed using the RT primer and a reverse primer designed to hybridize within the F gene (nucleotides 3894 to 3876 in the HMPV antigenome exclusive of GFP). An aliquot of each RT-PCR reaction was subjected to digestion with *Nhe*I. The RT-PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining. Lanes: 1 and 7, molecular weight marker; 2, a negative control in which RNA from the

25   recombinant HMPV-GFP-infected cell supernatant was assayed with the omission of RT enzyme; lanes 3 and 4, RT-PCR products from RNA representing recombinant HMPV-GFP and biologically-derived HMPV, respectively; lanes 5 and 6, RT-PCR products from RNA representing recombinant HMPV-GFP and biologically-derived HMPV, respectively, that had been subjected to digestion with *Nhe*I.

30   FIG. 12B is a digitized image of a nylon membrane illustrating Northern blot analysis of GFP mRNA expressed by the rHMPV-GFP virus. LLC-MK2 cells were mock-infected (lanes 1 and 5) or infected at an MOI of 3 PFU per cell with biologically-derived HMPV83 (lanes 2 and 6), rHMPV (lanes 3 and 7), or rHMPV-GFP (lanes 4 and 8). Three days later, total intracellular RNA was isolated, electrophoresed on 1% agarose-formaldehyde gels, transferred to charged nylon and

35   analyzed by hybridization to double-stranded ^{32}P-labeled DNA probe specific to the GFP (lanes 1-4) or M (lanes 5-8) gene. The identities and calculated sizes of individual RNA species are indicated.

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This showed that the GFP transcription cassette was expressed predominantly as a single mRNA of the appropriate size to be a monocistronic GFP mRNA.

FIG. 13 is a graph illustrating multi-step growth of the recovered recombinant HMPV (strain 83) (rHMPV) compared with biologically-derived HMPV 83. LLC-MK2 cells were inoculated
5   with 0.01 plaque forming units (pfu) per cell and incubated at 32°C in the presence of 5 µg/ml trypsin. Aliquots were taken at 24 h intervals, flash-frozen, and viral titers were measured by plaque assay and immunostaining with convalescent serum from HMPV-infected hamsters. Each time point was represented by two wells, and each virus titration was done in duplicate. Means are shown.

FIG. 14 is a graph illustrating multi-step growth of recombinant HMPV-GFP compared to
10   recombinant HMPV in LLC-MK2 cells, performed as described above for Figure 13. Two separate infections (a and b) were monitored for each virus, and each aliquot was titrated in duplicate and the mean is shown.

FIG. 15 is a diagram illustrating deletion of the SH and G genes singly and in combination
    from rHMPV and rHMPV-GFP (strain 83), each containing introduced *BsiWI* and *BsrGI* restriction
15   sites. The region of the antigenomic cDNA clone containing the putative SH and G ORFs is illustrated, together with proposed GS and GE transcription signals and proposed intergenic regions. *BsiWI* and *BsrGI* sites were introduced into the putative M2-SH and SH-G, respectively, intergenic regions of HMPV-GFP. In each case, the mutant sequence (nucleotide substitutions in small case) is shown below the wild type sequence. The restriction sites are numbered according to the position of
20   the first residue in the wild type HMPV sequence (SEQ ID NO: 1) exclusive of GFP, a convention that will be followed throughout this disclosure. The naturally-occurring *Acc65I* sequence also is shown. Note that cleavage of each of these restriction sites by its cognate enzyme leaves a compatible overhang, namely GTAC, that is compatible with each of the others. Thus, the genes can be readily deleted by cutting with the appropriate pair of enzymes and religating. This results in small changes
25   in the length of the relevant intergenic region, as noted. Each of these antigenomic cDNAs was successfully used to recover infectious mutant virus.

FIG. 16 is a digitized image of an agarose gel showing that the recovered recombinant HMPV-GFP ΔSH, ΔG and ΔSH/G viruses each contain a deletion of the appropriate size in the region of the SH and G genes. Following the procedure described above for Figure 12, viral RNA
30   was subjected to RT-PCR using primers that span the SH and G genes; specifically a positive sense primer designed to hybridize within the M2 and a negative sense primer designed to hybridize within the L gene. The RT-PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining. Lanes: 1 and 8, molecular weight markers; lane 3, a negative control in which RNA from recovered recombinant HMPV was subjected to the RT-PCR procedure with the omission
35   of RT enzyme; lanes 2 and 4-7, RT-PCR products representing the indicated viruses.

FIG. 17A is a graph illustrating multi-step growth of rHMPV-GFP-ΔSH, rHMPV-GFP-ΔG, and rHMPV-GFP-ΔSH/G compared to rHMPV and rHMPV-GFP, performed in LLC-MK2 cells as

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described above for Figure 13. Since the deletions had been introduced into rHMPV-GFP, rHMPV-GFP is the "wild type" equivalent for comparison.

FIG. 17B is a graph illustrating the efficiency of replication of biologically-derived HMPV83 and recombinantly-derived rHMPV, rHMPVΔSH, rHMPVΔG, and rHMPVΔSH/G in the
5   upper (nasal turbinates) and lower (lungs) respiratory tract of Golden Syrian hamsters. Animals in groups of 12 were infected intranasally with the following viruses at a dose of 5.0 X10^5 TCID_{50} per animal in 0.1 ml: biologically derived HMPV83, rHMPV, rHMPVΔSH, and rHMPVΔSH/G, and the following virus at 1.6 X 10^5 TCID_{50} per animal in 0.1 ml: rHMPVΔG. Six animals from each group were sacrificed on days 3 and 5 and the nasal turbinates and lungs were recovered and analyzed by
10   limiting dilution to determine the viral titer. This showed that the rHMPV replicated in vivo with an efficiency similar to that of its biologically-derived parent (HMPV83), that the replication of the rHMPVΔSH virus was not significantly reduced, and that the replication of the rHMPVΔG and ΔSH/G viruses were strongly reduced, although virus replication was detectable in each case.

FIG. 17C is a graph illustrating the protective efficacy of immunization with the indicated
15   HMPV deletion mutant viruses. Golden Syrian hamsters in groups of 6 were infected as described in Figure 17B and, 27 days later, serum samples were taken and analyzed to determine the titers of HMPV-neutralizing serum antibodies. These titers are shown at the bottom. The animals were then challenged on day 28 by the intranasal instillation of 5.0 log_{10} TCID_{50} per animal of biologically-derived wild type HMPV83. Three days later, the animals were sacrificed and the nasal turbinates
20   and lungs were harvested, homogenized, and analyzed by limiting dilution to determine virus titers. This showed that there was no detectable replication of the challenge virus in animals that has received rHMPV or rHMPVΔSH, whereas animals that initially had received rHMPVΔG or rHMPVΔSH/G had no detectable challenge virus replication in the lungs and had reduced replication in the nasal turbinates compared to the control that had not been previously infected.

25   FIG. 18A is a diagram illustrating ablation of the putative M2-2 ORF in infectious rHMPV and rHMPV-GFP (strain 83), each containing an introduced BsiWI site. The region of the antigenomic cDNA clone containing the putative M2-1 and M2-2 ORF is illustrated, with each ORF depicted by a solid horizontal line. Two nucleotide substitutions were made at positions 5236 and 5248 (SEQ ID NO: 1; numbered according to the complete antigenomic cDNA exclusive of GFP)
30   that ablate two potential translation initiation codons for the M2-2 ORF. A third substitution was made at position 5272 (SEQ ID NO: 1) that introduces an in-frame stop codon. In addition, nucleotides 5289-5440 (SEQ ID NO: 1) were deleted, removing most of the M2-2 ORF. The ΔM2-2 mutant was successfully recovered in both the rHMPV and rHMPV-GFP backbones and designated rHMPVΔM2-2 and rHMPV-GFPΔM2-2, respectively.

35   FIG. 18B is a graph illustrating evaluation of multi-step growth kinetics of the recovered viruses in vitro. The rHMPVΔM2-2 and rHMPV-GFPΔM2-2 viruses were compared with rHMPV and rHMPV-GFP with regard to multi-step growth kinetics and yield in LLC-MK2 cells as described

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above for Figure 13. rHMPVΔM2 clones #1 and #2 represent virus derived independently from two sister cDNA clones.

FIG. 18C is a digitized image of a nylon membrane illustrating Northern blot analysis of intracellular RNAs expressed by rHMPV-GFP and rHMPV-GFPΔM2-2. Replicate monolayers of

5   LLC-MK2 cells were infected at an MOI of 3 PFU per cell with either virus as indicated and incubated at 32°C. Monolayers were harvested at 9, 24, 36, 48, and 72 h post infection as indicated and processed for purification of intracellular RNA. The RNA preparations were analyzed by Northern blot hybridization with strand-specific riboprobes representing the N gene: the upper panels detect hybridization with the negative-sense probe and thus represent antigenome and N-related

10   mRNA, as indicated, and the bottom panels detect hybridization of a replicate set of gel lanes (that had been transferred in parallel onto the same membrane, which was then cut and the replicate sets hybridized separately) with positive-sense riboprobe and represent genome. Beneath the Northern blot are calculations from four experiments involving hybridization with a probe specific for N or for F, as indicated. In each experiment, the amount of total N-related or F-related mRNA for each time

15   point was divided by the amount of antigenome from a replicate gel lane (that had been transferred in parallel onto the same membrane, which was then cut and the replicas hybridized separately). Then, each time point for the rHMPV-GFPΔM2-2 virus was normalized relative to the corresponding time point for rHMPV-GFP as 1.0. Thus, this provides a comparison of the efficiency of mRNA expression for the ΔM2-2 mutant compared to wild type HMPV.

20   FIG. 19 is a diagram illustrating the introduction of single amino acid substitutions into the Cys3-His1 motif of the M2-1 protein of rHMPV-GFP (strain 83). This motif consists of three cysteine residues (C7, C15 and C21) and one histidine residue (H25); it is also found in RSV and other pneumoviruses, and thus represents a conserved pneumovirus motif. The amino acid sequence shown is from methionine (M) 1 to asparagine (N) 26 of the deduced complete M2-1 amino acid

25   sequence (SEQ ID NO: 4). Each of these mutants was successfully recovered as infectious recombinant virus.

FIG. 20 is a graph illustrating multi-step growth of derivatives of rHMPV-GFP containing mutations in the Cys3-His1 motif of the M2-1 ORF, compared to rHMPV-GFP and rHMPV, performed in LLC-MK2 cells as described above for Figure 13. The mutants involve single amino

30   acid point mutations in the Cys3-His1 motif: C7S, Y9S, C15S, N16S and H25S. The mutations were introduced into rHMPV-GFP, and thus rHMPV-GFP is the "wild type" or parental equivalent for comparison.

FIG. 21 is a diagram illustrating silencing of the M2-1 ORF in rHMPV-GFP (strain 83) by replacing the ATG translational start site (shaded) with a TAG translational termination codon, and

35   by replacing additional ATG triplets (underlined) in each reading frame with stop codons. The top line shows the nucleotide sequence of the upstream end of the M2-1 mRNA (corresponding to nucleotides 4711 to 4775 in the complete HMPV antigenome sequence; SEQ ID NO: 1). The second

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line shows nucleotide substitutions (lower case letters) introduced into the M2-1 sequence of
rHMPV-GFP to yield rHMPV-GFP-ΔM2-1. The third line shows the first 17 amino acids of the
HMPV M2-1 protein (SEQ ID NO: 4), and the fourth line shows coding changes introduced by the
point mutations in rHMPV-GFP-ΔM2-1, with termination codons in the M2-1 ORF indicated by

5   asterisks. Note that the next in-frame ATG in the M2-1 ORF is at codon 134 out of the total of 187
codons in the ORF (SEQ ID NO: 4). This mutant was successfully recovered as infectious
recombinant virus.

          FIG. 22A is a diagram illustrating deletion of the complete M2 gene in infectious rHMPV-
GFP (strain 83) containing an introduced *BsiWI* site. The region of the antigenomic cDNA clone

10   containing the putative M2-1 and M2-2 ORF is illustrated, with each ORF depicted by a solid
horizontal line. Nucleotides 4701 to 5459 (SEQ ID NO: 1), representing a total of 759 nucleotides,
were deleted, resulting in the mutant rHMPV-GFP-ΔM2(1+2). This mutant was successfully
recovered as infectious recombinant virus.

          FIG. 22B is a graph illustrating a comparison of the efficiency of growth of viruses with

15   alterations in the coding of M2-1 and/or M2-2 proteins in LLC-MK2 cells (upper panel), which are
competent for expressing type I interferons versus Vero cells (lower panel), which lack the interferon
structural genes. Cells were infected with 0.01 PFU per cell and incubated at 32°C in the presence of
5μg/ml trypsin. Aliquots were taken at 24 h intervals, flash-frozen, and viral titers were measured by
plaque assay and immunostaining with convalescent serum from hamsters that had been infected with

20   HMPV.

          FIG. 22C is a chart demonstrating increased sensitivity of HMPV ΔM2(1+2) and ΔM2-2
mutants to type I interferon. Replicate cultures of Vero cells were treated overnight with the indicated
amount of interferon per 1.5×10^6 cells. The cells were infected with the indicated amount of wild
type rHMPV-GFP, rHMPV-GFPΔM2(1+2), rHMPV-GFPΔM2-2, or rgRSV, the last being a

25   recombinant RSV that expresses the GFP gene. The GFP marker was used to visually monitor virus
growth during the experiment to judge the appropriate time of harvest, and is otherwise irrelevant to
this experiment. The cells were harvested on day 4 and the yield of each virus was determined and
compared with additional replicate cultures that had been mock-interferon treated, infected and
harvested in parallel. The results are expressed as the fold reduction of each interferon-treated culture

30   compared to its untreated counterpart.

          FIG. 23A is a diagram illustrating the construction of two rearrangements of the genes of
rHMPV-GFP (strain 83). The wild type gene order is shown on top for the M-L region of the
rHMPV-GFP antigenomic cDNA clone. The restriction sites shown are the ones introduced as
illustrated above in Figures 7 and 15 (the restriction sites are numbered according to the position of

35   the first residue in the wild type HMPV sequence, SEQ ID NO: 1, exclusive of GFP), and were used
to rearrange the order of the putative SH and G gene pair. Religations involving *NheI* were not
compatible with the other sites and necessitated fill-in of each end followed by blunt end ligation.

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Two examples of gene rearrangements are shown: in the mutant called "Order 1", the positions of the SH-G gene pair and the F-M2 gene pair were swapped, resulting in a local gene order that mimics that of RSV, namely M-SH-G-F-M2-L. This mutant was successfully recovered as infectious virus. In a subsequent mutant, "Order 2", the same RSV-like gene order was achieved, but the SH-G gene
5   pair was duplicated.

FIG. 23B is a graph illustrating the multi-step growth kinetics of one of these mutants, rHMPV-GFP-Order #1 compared to its direct parent rHMPV-GFP, as well as rHMPV, performed as described above for Figure 13. Clones #3 and #13 represent viruses derived independently from two sister cDNA clones.

10   FIG. 24A is a diagram illustrating shifting of the F and/or G genes of HMPV (strain 83) from their natural positions as the fourth and seventh genes, respectively, to promoter-proximal positions 1 or 2. As illustrated in the upper box, the putative F and G ORFs were engineered to be flanked by putative GS (bases 3054-3069 and 6219-6234 of SEQ ID NO: 1, respectively) and GE (bases 4685-4697 of SEQ ID NO: 1) signals and inserted individually into the HMPV antigenomic
15   cDNA clone. Sequence representing part of the 3' leader region (bases 23-41 of SEQ ID NO: 1) and the putative N GS signal and beginning of the putative N ORF (bases 42-66 of SEQ ID NO: 1) is shown. Note that the choice of transcription signals and insertion site was the same as for the rHMPV-GFP construct shown above in Figure 9, and places the inserted gene as the first in the gene order. As illustrated in the lower box, the putative F and G ORFs were inserted as a pair, in the order
20   G1-F2 or its converse F1-G2, using the same transcription signals and insertion site as in the single-gene rearrangements. Infectious virus was successfully recovered from each of the constructs. These viruses were designated rHMPV-F1, -G1, -F1G2, and G1F2, with the number indicating the position of the gene in the gene order. In the virus designation, the "-" symbol preceding the shifted gene indicates that the shifted gene was removed from its normal position in the gene order.

25   FIG. 24B is a graph illustrating multi-cycle growth kinetics of the recovered viruses *in vitro*. The recovered rHMPV-G1, F1, F1G2, and G1F2 viruses were compared to their rHMPV parent with regard to multi-step growth in LLC-MK2 cells as described above for Figure 13.

FIG. 25A is a diagram showing representations of rHMPV in which one or more extra copies of the F and/or G gene was placed in promoter proximal positions 1, 2 or 3 in addition to the F
30   and/or G gene present in the normal genome position. The G or F genes were inserted individually into promoter proximal position 1 (rHMPV+G1 or +F1), or were inserted as a pair into positions 1 and 2 in each of the two possible orders (rHMPV+G1F2 or +F1G2), or were inserted as the pair G1F2 with an additional copy of F in the third position (rHMPV+G1F2,3). The detailed structure of the gene insertions were as shown in Figure 24A. In the designations, the "+" symbol before the
35   named gene or genes indicates that it/they were additional to the one or ones in the normal position. The added genes are shaded.

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FIG. 25B is a graph illustrating multi-cycle growth kinetics *in vitro*. The recovered viruses were compared to their rHMPV parent and to rHMPV-GFP with regard to multi-step growth in LLC-MK2 cells as described above for Figure 13.

FIG. 25C is a digitized image of a nylon membrane illustrating Northern blot analysis of
5   intracellular mRNAs expressed by rHMPV+G1F23. LLC-MK2 cells were mock-infected (lanes 1, 5 and 9) or infected at an MOI of 3 PFU per cell with HMPV83 (lanes 2, 6 and 10), rHMPV (lanes 3, 7 and 11), or rHMPV+G1F23 (lanes 4, 8 and 12). Three days later, total intracellular RNA was isolated, electrophoresed on 1% agarose-formaldehyde gels, transferred to charged nylon and analyzed by hybridization to double-stranded ^{32}P-labeled DNA probe specific to the F (lanes 1-4), G
10   (lanes 5-8), or M (lanes 9-12) gene. The identities and calculated sizes of individual RNA species are indicated.

FIGS. 26A-26F are diagrams illustrating amino acid locations in the strain 83 HMPV L protein (SEQ ID NO: 7) that are targets for mutagenesis based on mapping of attenuating mutations in a heterologous virus (HRSVA2, SEQ ID NO: 13; HPIV3, SEQ ID NO: 14; HPIV1, SEQ ID NO:
15   15; or BPIV3, SEQ ID NO: 16, as indicated). HMPV strain 001, SEQ ID NO: 12, is included in the alignments for comparison. Numbers at the left indicate the positional reference of the first residue in the partial amino acid sequence shown, and numbers to the right indicate the positional reference number of the terminal residue in the partial sequence. The more highly conserved or similar residues are denoted by shading. Dashes indicate a gap in the particular sequence introduce to
20   maximize the alignment.

FIG. 27 is a graph illustrating multi-step growth of rHMPV-GFP containing the F456L mutation (see Figure 26A), compared to rHMPV, performed in LLC-MK2 cells as described above for Figure 13. Clones #3 and #7 represent viruses derived independently from two sister cDNA clones.

25   FIG. 28 is a diagram illustrating the GS signal of the M2 gene of wild type (WT) RSV (SEQ ID NO: 31), and a highly-attenuating T to C nucleotide substitution at position nine in the GS signal of the M2 gene of the attenuated cpts248/404 RSV mutant (Whitehead *et al.*, J. Virol. 72:4467-4471, 1998). There is a corresponding T residue that is highly conserved among all of the putative GS signals of HMPV. The individual GS motifs are named according to the putative ORF that each
30   precedes (SEQ ID NO: 1, bases 41-57 for N; bases 1249-1265 for P; bases 2166-2182 for M; bases 3053-3069 for F; bases 4710-4726 for M2; bases 5467-5483 for SH; bases 6218-6234 for G; and bases 7119-7135 for L). The sequence at the bottom (SEQ ID NO: 32) indicates in upper case letters those positions in the putative HMPV GS signal that are exactly conserved among all of the signal in strain 83, which includes the T at position nine.

35   FIG. 29 is a diagrammatic representation of the genome structures of biologically-derived HMPV strains 83 and CAN98-75 (hereafter referred to as 75 or CAN75), representing the two proposed genetic HMPV subgroups and thus illustrating the diversity that must be considered in

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designing an HMPV immunogenic composition. Individual genes are indicated by boxes, with gene
lengths and boundaries within the complete genomic sequence given in nucleotides together with the
unmodified amino acid length (italics) of the deduced protein (in the case of M2, two predicted
proteins, M2-1 and M2-2). The nucleotides lengths of the extragenic 3' leader, 5' trailer and
5   intergenic regions are underlined.

FIG. 30 is a diagram illustrating the construction of chimeric viruses constructed with genes
of HMPV and APV, which represent potential vaccine candidates. Part A illustrates the genome of
HMPV83 and derivatives in which the N, P or M ORF is replaced individually by its APV
counterpart (unshaded boxes). Parts B, C and D illustrate the structures of cDNAs containing the N, P
10   and M genes, respectively of HMPV (upper line in each box) compared to a version containing the
indicated substituted APV ORF (lower line in each box). Each ORF is shown as a shaded box and is
flanked by HMPV GS (SEQ ID NO: 1, bases 12-57 for the leader/N GS signal; bases 1235-1265 for
the N GE/P GS·signal; and bases 2146-2182 for the P GE/M GS signal) and GE (SEQ ID NO: 1,
bases 1235-1242 for the partial N GE signal; bases 2146-2151 for the partial P GE signal; and bases
15   3007-3037 for the M GE signal) transcription signals (boxed). In the case of the APV M gene, 64 nt
of noncoding sequence that separates the ORF from the GE signal have been deleted (-64 nts nc). The
GE and GS signals in turn are flanked by restriction sites selected from the following list, as
indicated: *BbsI, BsmBI, BfuAI, BbsI*. Each of these sites consists of a recognition sequence
(underlined) and has the property of cutting in a sequence-independent fashion at a second site
20   outside of the recognition site (underlined tetranucleotide, bold) to leave a 4-nt 5'-protruding
overhang. Here, the overhangs were TAAT or GTAG, as indicated, which matches the natural HMPV
assignments at these positions. Part E illustrates a novel assembly strategy that utilizes the 4-nt
overhangs, plus the flanking *MluI* and *NheI* sites, for ligation of the N, P and M cDNAs
simultaneously into the HMPV backbone. By this strategy, this can be used to mix HMPV and APV
25   N, P and M genes in whatever combinations desired to make replacements of single HMPV genes (as
in part A) or to replace two or three genes simultaneously.

FIG. 31 is a diagram showing the comparison of the predicted gene boundaries, cis-acting
GS and GE signals, and intergenic regions of HMPV strains 83 (SEQ ID NO: 1) and 75 (SEQ ID
NO: 2), shown as positive-sense sequence. The top alignment shows the leader/N gene boundary,
30   specifically the last 16-18 nucleotides of the leader region followed by the first 15 nucleotides of the
N gene. The second alignment shows the N/P gene boundary, specifically the last 15 nucleotides of
the N gene, followed by the adjoining intergenic region, followed by the first 15 nucleotides of the P
gene. Subsequent alignments show the P/M, M/F, F/M2, M2/SH, SH/G, G/L, and L/trailer
boundaries. Conserved sequence motifs at the end (gene end) and beginning (gene start) of each gene
35   are indicated in bold upper case, and a consensus is given below (SEQ ID NOs: 34 and 35).
Positions within these conserved motifs are numbered. Translational stop and start codons are
underlined. Intergenic sequences are shown: in the case of the longer intergenic regions, only the

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first 5 nucleotides on the upstream and downstream ends are shown, and the number of nucleotides not shown is indicated. For each assignment in the consensus sequence, no more than two of the sequence pairs (HMPV 83 versus 75) could have a heterologous assignment in both subgroup sequences.

5   FIG. 32 is a chart illustrating the percent amino acid and nucleotide sequence identity (the latter given in parentheses) between the predicted proteins and ORFs of: HMPV 83 versus HMPV 75, representing a comparison across subgroups; HMPV83 versus HMPV 00-1, representing a comparison within a subgroup; and RSV A2 versus RSV B1, representing a comparison across the two well-characterized RSV antigenic subgroups A and B, which serve here as a benchmark for

10   assessing the magnitude and anticipated significance of the differences in the HMPV subgroups. Amino acid sequence identities were calculated based on the complete predicted proteins; in the case of G and SH, overhangs on the carboxy-terminal side of alignments due to length differences were not included in the calculations. Nucleotide sequence identities for the corresponding ORFs are shown in parentheses and are based on the protein-coding sequence exclusive of flanking noncoding

15   sequence. For comparison, the HMPV intergenic regions were 48% identical between subgroups and noncoding gene sequences exclusive of conserved transcription signals were 54% identical, compared to values of 42% and approximately 50% for RSV (Johnson et al., J. Gen. Virol. 69:2901-2906, 1988).

          FIGS. 33A-33B are diagrams showing the alignment of the amino acid sequences of the SH

20   (Figure 33A) and G (Figure 33B) proteins of HMPV strains 75 (SEQ ID NOs: 8 and 9, respectively), 83 (SEQ ID NOs: 5 and 6, respectively) and 00-1 (SEQ ID NOs: 10 and 11, respectively). This includes comparison between (75 versus 83) and within (83 versus 00-1) the proposed HMPV genetic subgroups. For strains 75 and 00-1, assignments that differ from that of 83 are shown; dashes indicate gaps introduced to maximize the alignment or to denote the absence of corresponding amino

25   acids. Stars underneath each alignment denote amino acid identity among all three sequences; small dots indicate amino acid similarity among all three. Proposed signal/transmembrane domains are boxed. Motifs for N-linked carbohydrate are underlined (N-X-T/S, where X is not proline). In panel A, cysteine residues conserved among all three SH proteins are indicated with large dots, and potential sites for O-linked glycosylation (Hansen et al., Glycoconj. J. 15:115-130, 1998) of the SH

30   proteins are as follows: HMPV 83, 75, 77, 78, 81; HMPV 75, 78, 79, 81; 00-1, 77, 78, 81. The ectodomain of the G protein of each virus contained more than 40 potential acceptor sites for O-linked carbohydrate.

          FIG. 34 is a diagram illustrating the design of a chimeric HMPV virus bearing antigens specific to HMPV strains that represent two different antigenic subgroups of HMPV. The strain 83

35   antigenomic cDNA clone was used as the backbone (sequence representing part of the 3' leader region (bases 23-41 of SEQ ID NO: 1) and the putative N GS signal and beginning of the putative N ORF (bases 42-66 of SEQ ID NO: 1) is shown), and a transcription cassette was designed that

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consists of the ORF encoding the putative G, F, or SH surface proteins of strain 75 under the control
of transcription signals from strain 83 (which including bases 42-57 of SEQ ID NO: 1, representing
the conserved putative N GS signal, and bases 4685-4697 of SEQ ID NO: 1, representing the putative
F GE signal). This transcription cassette is then inserted into the strain 83 backbone at a promoter-
5   proximal site. This strategy for constructing a bivalent immunogenic construct employs the same
strain 83 transcription signals and insertion site as shown for the HMPV-GFP construct in Figure 9.

FIG. 35 is a graph illustrating the daily level of replication of HMPV strains 75 and 83 in
the upper and lower respiratory tract of African green monkeys. Mean daily virus titers in the
nasopharyngeal (upper panel) or tracheal lavage (lower panel) specimens obtained on the indicated
10 . day post-inoculation from animals infected simultaneously by the intranasal and intratracheal routes
with 105 TCID50 of the indicated virus: strain 75 (●), strain 83 (■).

FIGS. 36A-36B are a diagram and digitized images showing the construction and
expression of a recombinant HPIV1 vector expressing the F protein of HMPV CAN83 (rHPIV1-F$_{83}$).
The HPIV1 genome was modified by the creation of an *Mlu*I restriction site (HPIV1 nt 113-118) one
15   nucleotide prior to the translational start codon of the N ORF (HPIV1 nt 119-121). The F ORF of
HMPV strain CAN83 (1620 nt in length and encoding a 539 aa polypeptide; bases 3054-4697 of SEQ
ID NO: 1) was engineered by PCR to be followed by the tetranucleotide TAAT and an HPIV1 gene
junction consisting of a GE signal, CTT intergenic region, and a GS signal. The length of the entire
cassette was 1656 nt and was designed, upon insertion into the *Mlu*I site, to conform to the rule of six
20   and to maintain the HPIV1 GS signal sequence phasing. GS and GE signals are indicated (FIG.
36A). A portion of the predicted viral promoter that lies within the N gene is shown (FIG. 36A).
LLC-MK2 cells were infected with rHPIV-F83, HMPV CAN83, rHPIV1 or mock-infected, as
indicated, incubated for 72 hr, and analyzed by indirect immunofluorescence using anti-HMPV
polyclonal hamster serum or a mixture of two mouse monoclonal antibodies to the HPIV1 HN
25   protein, as indicated (Fig. 36B).

FIGS. 37A-37D are the complete sequence of the genome of biologically-derived strain 83,
shown as positive sense DNA (SEQ ID NO: 1). See also, GenBank Accession No. AY297749.

FIGS. 38A-38D are the complete sequence of the genome of recombinant strain 83 bearing
a transcription cassette expressing GFP, which recombinant strain exemplifies rHMPV-GFP of the
30   current disclosure (SEQ ID NO: 3).

FIGS. 39A-39D are the complete sequence of the genome of biologically-derived,
biologically-cloned HMPV CAN75 (SEQ ID NO: 2). See also, GenBank Accession No. AY297748.

SEQUENCE LISTING

35   The nucleic and amino acid sequences listed in the accompanying sequence listing are
shown using standard letter abbreviations for nucleotide bases, and three letter code for amino acids,
as defined in 37 C.F.R. 1.822. Only one strand of each nucleic acid sequence is shown, but the

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complementary strand is understood as included by any reference to the displayed strand. In the accompanying sequence listing:

SEQ ID NO: 1 shows the nucleic acid sequence of the genome of biologically-derived HMPV (strain 83) as positive sense DNA.

SEQ ID NO: 2 shows the nucleic acid sequence of the genome of biologically-derived HMPV (strain 75) as positive sense DNA.

SEQ ID NO: 3 shows the nucleic acid sequence of the genome of recombinant HMPV (strain 83) with a transcription cassette expressing GFP as positive sense DNA.

SEQ ID NO: 4 shows the amino acid sequence of the HMPV (strain 83) M2-1 protein.

SEQ ID NO: 5 shows the amino acid sequence of the HMPV (strain 83) SH protein.

SEQ ID NO: 6 shows the amino acid sequence of the HMPV (strain 83) G protein.

SEQ ID NO: 7 shows the amino acid sequence of the HMPV (strain 83) L protein.

SEQ ID NO: 8 shows the amino acid sequence of the HMPV (strain 75) SH protein.

SEQ ID NO: 9 shows the amino acid sequence of the HMPV (strain 75) G protein.

SEQ ID NO: 10 shows the amino acid sequence of the HMPV (strain 00-1) SH protein.

SEQ ID NO: 11 shows the amino acid sequence of the HMPV (strain 00-1) G protein.

SEQ ID NO: 12 shows the amino acid sequence of the HMPV (strain 00-1) L protein.

SEQ ID NO: 13 shows the amino acid sequence of the HRSVA2 L protein.

SEQ ID NO: 14 shows the amino acid sequence of the HPIV3 L protein.

SEQ ID NO: 15 shows the amino acid sequence of the HPIV1 L protein.

SEQ ID NO: 16 shows the amino acid sequence of the BPIV1 L protein.

SEQ ID NO: 17 shows the nucleic acid sequence of the HMPV (strain 00-1) 3' leader.

SEQ ID NO: 18 shows the nucleic acid sequence of the HMPV (strain 83) 3' leader.

SEQ ID NO: 19 shows the nucleic acid sequence of the APV 3' leader.

SEQ ID NO: 20 shows the nucleic acid sequence of the RSV 3' leader.

SEQ ID NO: 21 shows the nucleic acid sequence of the HMPV (strain 00-1) 5' trailer.

SEQ ID NO: 22 shows the nucleic acid sequence of the HMPV (strain 83) 5' trailer.

SEQ ID NO: 23 shows the nucleic acid sequence of the APV 5' trailer.

SEQ ID NO: 24 shows the nucleic acid sequence of the RSV 5' trailer.

SEQ ID NO: 25 shows the nucleic acid sequence of the HMPV (strain 83) GS consensus sequence.

SEQ ID NO: 26 shows the nucleic acid sequence of the HMPV (strain 83) GS consensus sequence, with alternative nucleic acid assignments.

SEQ ID NO: 27 shows the nucleic acid sequence of the HMPV (strain 83) GS consensus sequence, with alternative nucleic acid assignments.

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SEQ ID NO: 28 shows the nucleic acid sequence of the HMPV (strain 83) GE consensus sequence.

SEQ ID NO: 29 shows the nucleic acid sequence of the HMPV (strain 83) GE consensus sequence, with alternative nucleic acid assignments.

5   SEQ ID NO: 30 shows the nucleic acid sequence of the HMPV (strain 83) GE consensus sequence, with alternative nucleic acid assignments.

SEQ ID NO: 31 shows the nucleic acid sequence of the RSV M2 GS sequence.

SEQ ID NO: 32 shows the nucleic acid sequence of the HMPV (strain 83) GS consensus sequence, with uniformly conserved bases.

10   SEQ ID NO: 33 shows the nucleic acid sequence of a polylinker used in the cloning and expression of rHMPV.

SEQ ID NO: 34 shows the nucleic acid sequence of the HMPV (strain 83/75) GE consensus sequence.

SEQ ID NO: 35 shows the nucleic acid sequence of the HMPV (strain 83/75) GS consensus

15   sequence.

SEQ ID NO: 36 shows the nucleic acid sequence of the genome of HMPV (strain 00-1) as positive sense DNA.

20   **DETAILED DESCRIPTION OF SEVERAL EMBODIMENTS**

In one embodiment, methods and compositions are provided for producing an infectious, self-replicating, recombinant HMPV from one or more isolated polynucleotide molecules encoding viral sequences. The methods and compositions generally involve coexpressing in a cell or cell-free system one or more expression vectors comprising a polynucleotide molecule that encodes a partial

25   or complete, recombinant HMPV genome or antigenome and one or more polynucleotide molecules encoding HMPV N, P, and L proteins, so as to produce an infectious HMPV particle. In certain embodiments, the methods and compositions for producing the recombinant HMPV further include expression of the M2-1 gene.

Typically, the polynucleotide molecule that encodes the recombinant HMPV genome or

30   antigenome is a cDNA. Thus, disclosed herein are polynucleotides such as cDNAs and their equivalents that encode a recombinant HMPV. Expression vectors and constructs that incorporate a polynucleotide molecule encoding a recombinant HMPV genome or antigenome are also disclosed herein.

The HMPV genome or antigenome, and the N, P, M2-1 and L proteins can all be produced

35   from a single expression vector. However, the genome or antigenome can be produced by a separate expression vector, and the N, P, M2-1 and L proteins can be produced by one, two, or more additional expression vectors. One or more of the N, P, M2-1 and L proteins can be supplied by

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expression of a recombinant HMPV genome or antigenome. These proteins can also be supplied by coinfection with the same or different HMPV. Thus, in several embodiments, one or more of the N, P, M2-1 and L proteins are from a heterologous HMPV.

5   Infectious, recombinant, self-replicating viral particles are disclosed that are produced according to the foregoing methods. These particles include complete viruses as well as viruses that lack one or more non-essential proteins or non-essential portions (for example, a cytoplasmic, transmembrane or extracellular domain) of a viral protein. Viruses of the current disclosure that lack one or more such non-essential components (for example, a gene or genome segment from the HMPV SH, G, M2-2 open reading frame (ORF) or comparable accessory ORFs, or a segment of one

10   or more other ORFs, or an intergenic or other non-coding or non-essential genome component) are referred to herein as incomplete viruses or "subviral particles." Exemplary subviral particles can lack any selected structural element, including a gene, gene segment, protein, protein functional domain, etc. that is present in a complete virus (that is, an assembled virion including a complete genome or antigenome, nucleocapsid and envelope). For example, a subviral particle of the current disclosure

15   can include an infectious nucleocapsid containing a genome or antigenome, and the products of the N, P, and L genes. Other subviral particles are produced by partial or complete deletions or substitutions of non-essential genes and/or their products among other non-essential structural elements.

        Complete viruses and subviral particles produced by the methods disclosed herein are

20   typically infectious and self-replicative through multiple rounds of replication in a mammalian host amenable to infection by HMPV. These hosts include various *in vitro* mammalian cell populations, *in vivo* animal models widely known and accepted in the art as reasonably predictive of HMPV activity in humans (including, mice, hamsters, cotton rats, non-human primates including African green monkeys and chimpanzees), and humans, including seronegative and seropositive infants,

25   children, juveniles, and adults. However, viruses and subviral particles also can be produced that are highly defective for replication *in vivo*.

        In one embodiment, the polynucleotide molecule encodes a sequence of a wild type HMPV (either the genome or the antigenome). The term "wild type" refers to a gene or gene product which has the characteristics of that gene or gene product when isolated from a naturally occurring source.

30   A wild-type gene is that which is most frequently observed in a population and is thus arbitrarily designed the "normal" or "wild-type" form of the gene. In another embodiment, the genome or antigenome can include one or more mutations from a biologically derived mutant HMPV, or any combination of recombinantly-introduced mutations; including one or more polynucleotide insertions, deletions, substitutions, or rearrangements that is/are selected to yield desired phenotypic

35   effects in the recombinant virus.

        Thus, the recombinant HMPV genome or antigenome can be engineered using the methods disclosed herein to incorporate a recombinantly-introduced restriction site marker, or a translationally

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silent point mutation for handling or marking purposes. In other embodiments, the polynucleotide molecule encoding the recombinant HMPV genome or antigenome can incorporate one or more recombinantly-introduced attenuating mutations. In specific examples, the recombinant HMPV genome or antigenome incorporates one or more recombinantly-introduced, temperature sensitive (ts) or host range (hr) attenuating (att) mutations.

The recombinant HMPV genome or antigenome can incorporate one or more attenuating mutations identified in a biologically derived mutant HMPV strain, or in another mutant nonsegmented negative stranded RNA virus. For example, a mutation in a L, M, N, P, M2-1, M2-2, SH, F, or G protein, or in an extragenic sequence selected from a 3' leader or in a control signal such as the M2 GS sequence. Where the mutation is of one or more particular amino acid residues, the recombinant HMPV genome or antigenome can incorporate multiple nucleotide changes in the codons specifying the mutation to stabilize the modification against reversion.

The recombinant HMPV genome or antigenome can include an additional nucleotide modification specifying a phenotypic change, such as an alteration in growth characteristics, attenuation, temperature-sensitivity, cold-adaptation, plaque size, host-range restriction, or a change in immunogenicity. These additional modifications can alter one or more of the HMPV N, P, M, SH, G, F, M2-1, M2-2 and/or L genes and/or a 3' leader, 5' trailer, a cis-acting sequence such as a GS or GE sequence, and/or intergenic region within the HMPV genome or antigenome. For example, one or more HMPV genes can be deleted in whole or in part, or expression of the genes can be reduced or ablated by a frameshift mutation, by a mutation that alters a translation start site, by introduction of one or more stop codons in an ORF of the gene, or by a mutation in a transcription signal. In several embodiments, the recombinant HMPV genome or antigenome is modified by a partial or complete deletion of the HMPV SH, G, or M2-2 gene or ORFs, or one or more nucleotide changes that reduces or ablates expression of one or more HMPV genes yet yields a viable, replication competent, infectious viral construct. In other embodiments, the recombinant HMPV genome or antigenome is modified to encode a non-HMPV molecule such as a cytokine, a T-helper epitope, a restriction site marker, or a protein of a microbial pathogen capable of eliciting a protective immune response in a mammalian host.

A recombinant HMPV genome or antigenome can include a partial or complete HMPV "vector" genome or antigenome combined with one or more heterologous genes or genome segments (nucleic acid sequences) encoding one or more antigenic determinants of one or more heterologous pathogens to form a chimeric HMPV genome or antigenome. The heterologous genes or genome segments encoding the antigenic determinants can be added as supernumerary genes or genome segments adjacent to or within a noncoding region of the partial or complete HMPV vector genome or antigenome, or can be substituted for one or more counterpart genes or genome segments in a partial HMPV vector genome or antigenome. The heterologous genes or genome segments can include one or more heterologous coding sequences and/or one or more heterologous regulatory

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elements comprising an extragenic 3' leader or 5' trailer region, a GS signal, GE signal, editing
region, translational start site, intergenic region, or a 3' or 5' non-coding region.

In additional embodiments, the heterologous pathogen is one or more heterologous
pneumoviruses (for example, a heterologous HMPV or RSV) and the heterologous genes or genome
5   segments encodes one or more HMPV or RSV N, P, M, SH, G, F, M2-1, M2-2 and/or L proteins or
fragments thereof. Thus, the antigenic determinants can be from a heterologous HMPV or RSV G,
SH and F glycoproteins, and antigenic domains, fragments and epitopes thereof, that is/are added to
or substituted within the partial or complete HMPV genome or antigenome. In several examples,
genes encoding G, SH and F glycoproteins of a heterologous HMPV or RSV are substituted for
10   counterpart HMPV G, SH and F genes in a partial HMPV vector genome or antigenome. In
additional examples, genes encoding the G, SH, and F glycoproteins of a heterologous HMPV or
RSV are expressed in addition to the HMPV sequences. In this manner, a plurality of heterologous
genes or genome segments encoding antigenic determinants of multiple heterologous pneumoviruses
can be added to or incorporated within the partial or complete HMPV vector genome or antigenome.
15   In other embodiments, one or more genes from APV or a related pneumovirus exhibiting a host range
restriction in humans is used to replace the corresponding genes in humans to achieve an attenuated
derivative and other improved properties.

The disclosed recombinant human metapneumoviruses (HMPVs) can be used to generate a
desired immune response against one or more HMPVs, or against HMPV and one or more non-
20   HMPV pathogens, in a subject susceptible to infection. Recombinant HMPV as disclosed herein are
capable of eliciting a mono- or poly-specific immune response in an infected mammalian host, yet are
sufficiently attenuated so as to not cause unacceptable symptoms of disease in the immunized host.
The attenuated viruses, including complete viruses and subviral particles, can be present in a cell
culture supernatant, isolated from the culture, or partially or completely purified. The virus can also
25   be lyophilized, and can be combined with a variety of other components for storage or delivery to a
host, as desired.

Immunogenic compositions are also disclosed herein that include a physiologically
acceptable carrier and/or adjuvant and an isolated attenuated recombinant HMPV virus. In one
embodiment, the immunogenic composition includes a recombinant HMPV having at least one, at
30   least two, or more attenuating mutations or other nucleotide modifications that specify a suitable
balance of attenuation and immunogenicity. In specific examples, the immunogenic composition can
be formulated in a dose of 10^3 to 10^7 PFU of attenuated virus. The immunogenic composition can
include an attenuated recombinant HMPV that elicits an immune response against a single HMPV
strain or against multiple HMPV strains or serotypes or other pathogens such as RSV and/or HPIV.
35   In this regard, recombinant HMPV can be combined in formulations with other HMPV strains, or
with other candidate viruses such as a live attenuated RSV. Methods are also disclosed herein for
stimulating the immune system of a mammalian subject to elicit an immune response against one or

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more HMPVs, or against HMPV and a non-HMPV pathogen. Thus, a method is provided herein for inducing an immune response against a single HMPV, against multiple HMPVs, or against one or more HMPVs and a non-HMPV pathogen such as RSV.

The disclosed recombinant HMPVs can be used to elicit a monospecific immune response or
5   a polyspecific immune response against multiple HMPVs, or against one or more HMPVs and a non-HMPV pathogen. Alternatively, recombinant HMPV having different immunogenic characteristics can be combined in a mixture or administered separately in a coordinated treatment protocol to elicit an immune response against one HMPV, against multiple HMPVs, or against one or more HMPVs and a non-HMPV pathogen such as RSV. In one example, the immunogenic compositions are
10   administered to the upper respiratory tract, for example, by spray, droplet or aerosol.

An operable set of viral open reading frames (ORFs) sufficient to direct viral transcription and RNA replication are disclosed herein. Diagnostic assays using this set of ORFs are provided that determine viral activities based either on infectious virus or on the expression of isolated polynucleotide molecules encoding viral sequences. In one example, an infectious HMPV
15   recombinant virus is disclosed herein that expresses a detectable marker or label, such as a recombinant HMPV expressing the jellyfish green fluorescent protein (GFP). These and related tools are effective, for example, to determine and characterize HMPV infection *in vitro* and *in vivo*. In one exemplary embodiment, recombinant HMPV expressing a detectable label can be utilized in assays and related compositions for the detection of HMPV-neutralizing antibodies in biological specimens
20   (for example, serum of patients at risk of HMPV infection or presenting with respiratory symptoms). In additional embodiments the recombinant HMPV expressing a detectable label can be used to screen compounds for antiviral activity.

Unless otherwise noted, technical terms are used according to conventional usage. Definitions of common terms in molecular biology may be found in Benjamin Lewin, *Genes VII,*
25   published by Oxford University Press, 2000 (ISBN 019879276X); Kendrew *et al.* (eds.), *The Encyclopedia of Molecular Biology,* published by Blackwell Publishers, 1994 (ISBN 0632021829); and Robert A. Meyers (ed.), *Molecular Biology and Biotechnology: a Comprehensive Desk Reference,* published by Wiley, John & Sons, Inc., 1995 (ISBN 0471186341); and other similar references.
30   As used herein, the singular terms "a," "an," and "the" include plural referents unless context clearly indicates otherwise. Similarly, the word "or" is intended to include "and" unless the context clearly indicates otherwise. Also, as used herein, the term "comprises" means "includes." Hence "comprising A or B" means including A, B, or A and B. It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids
35   or polypeptides are approximate, and are provided for description. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications,

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patents, and other references mentioned herein are incorporated by reference in their entirety. In case
of conflict, the present specification, including explanations of terms, will control. In addition, the
materials, methods, and examples are illustrative only and not intended to be limiting.

5
            As described above, methods and compositions for the production and use of novel,
recombinant HMPVs are provided herein. The recombinant HMPVs are infectious and immunogenic
in humans and other mammals and are useful as immunogenic compositions to produce immune
responses against one or more HMPVs or other pneumoviruses. Thus, the recombinant HMPVs can
10   be used to produce an immune response to, for example, RSV and/or one or more HMPV or other
pneumoviral strains, serotypes, or subgroups. Chimeric HMPVs are provided herein that can be used
to elicit an immune response against a selected HMPV and one or more additional pathogens, for
example against multiple HMPVs or against a HMPV and a non-HMPV virus such as RSV, or
parainfluenza virus (PIV). The immune response elicited can involve either or both humoral and/or
15   cell mediated responses. The HMPVs can be attenuated to yield a desired balance of attenuation and
immunogenicity.
            Methods are provided herein for designing and producing attenuated, HMPVs that are useful
as agents for eliciting a desired immune response against HMPV and other pathogens. Thus, using
the methods disclosed herein, recombinant HMPVs can be produced that have a defined genome
20   sequence and predictable characteristics.
            An exemplary recombinant HMPV (rHMPV) includes a recombinant HMPV genome or
antigenome and encodes (or the antisense of which encodes) HMPV major nucleocapsid (N) protein,
nucleocapsid phosphoprotein (P), and large polymerase protein (L) (see FIG. 1 and exemplary
sequence information provided in FIG. 37). In additional embodiments, the rHMPV can incorporate
25   a recombinant HMPV genome or antigenome, N, P, and L proteins, and a HMPV M2-1 protein. In
further embodiments, one or more of the N, P, L, and/or M2-1 proteins can be a mutant protein or
partial protein, or include a corresponding homologous protein or protein fragment of a heterologous
HMPV or non-HMPV virus such as APV. One or more additional HMPV proteins can be
coexpressed with the recombinant HMPV genome or antigenome, in various combinations, to
30   provide a range of infectious viruses. As used herein the term "recombinant HMPV" or "rHMPV"
includes recombinantly produced subviral particles lacking one or more non-essential viral
components, complete viruses having all native viral components, and viruses containing
supernumerary proteins, antigenic determinants or other additional components. A recombinant virus
is produced entirely from cloned cDNA.
35   As set forth in the Examples below, a complete consensus sequence was determined for the
genomic RNA of an exemplary wild type strain of HMPV. The sequence thus identified was used to
generate a full-length antigenomic cDNA and to recover a wild type rHMPV. The biological

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properties of rHMPV *in vitro* and *in vivo* demonstrates that the exemplary rHMPV sequence corresponds to a wild type virus. This critical finding demonstrates that the recombinant HMPV sequence disclosed herein is that of an authentic wild type virus. This rHMPV serves as a novel substrate for recombinant introduction of attenuating mutations for the generation of live–attenuated

5   HMPV and HMPV-based chimeric and vectors that can be used for immunogenic compositions.

As disclosed herein, infectious recombinant HMPVs can be produced by a recombinant coexpression system that permits introduction of defined changes into the recombinant HMPV and provides for the generation, with high frequency and fidelity, of HMPV having a defined genome sequence. These modifications are useful in a wide variety of applications, including the

10   development of live attenuated viral strains bearing predetermined, defined attenuating mutations. Infectious HMPV can be produced by intracellular or cell-free coexpression of one or more isolated polynucleotide molecules that encode the HMPV genome or antigenome RNA, together with one or more polynucleotides encoding viral proteins to generate a transcribing, replicating nucleocapsid. An "infectious virus" is a viral particle that has the ability to deliver its genome to the cytoplasm of a

15   host cell.

The cDNAs encoding a HMPV genome or antigenome are constructed for intracellular or *in vitro* coexpression with the selected viral proteins to form infectious HMPV. A "HMPV antigenome" is an isolated positive-sense polynucleotide molecule which serves as a template for synthesis of progeny HMPV genome. In one embodiment a cDNA is constructed which is a positive-

20   sense version of the HMPV genome that corresponds to the replicative intermediate RNA, or antigenome. This minimizes the possibility of hybridizing with positive-sense transcripts of complementing sequences encoding proteins necessary to generate a transcribing, replicating nucleocapsid. A "replication competent virus" is a viral particle capable of initiating an infection that produces viral progeny.

25   In some embodiments the genome or antigenome of a recombinant HMPV contains only those genes or portions thereof necessary to render the viral or subviral particles encoded thereby infectious. Further, the genes or portions thereof may be provided by more than one polynucleotide molecule, that is, a gene may be provided by complementation or the like from a separate nucleotide molecule. In other embodiments, the HMPV genome or antigenome encodes all functions necessary

30   for viral growth, replication, and infection without the participation of a helper virus or viral function provided by a plasmid or helper cell line.

A "recombinant HMPV particle" is a HMPV or HMPV-like viral or subviral particle derived directly or indirectly from a recombinant expression system or propagated from virus or subviral particles produced there from. The recombinant expression system can employ a recombinant

35   expression vector which includes an operably linked transcriptional unit comprising an assembly of at least a genetic element or elements having a regulatory role in HMPV gene expression, for example, a promoter, a structural or coding sequence which is transcribed into HMPV RNA, and appropriate

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transcription initiation and termination sequences. A first nucleic acid sequence is "operably linked" with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence.

5   To produce infectious HMPV from a cDNA-expressed HMPV genome or antigenome, the genome or antigenome is coexpressed with those HMPV or heterologous viral proteins necessary to produce a nucleocapsid capable of RNA replication, and render progeny nucleocapsids competent for both RNA replication and transcription. Transcription by the genome nucleocapsid provides the other HMPV proteins and initiates a productive infection. Alternatively, additional HMPV proteins useful for a productive infection can be supplied by coexpression.

10   In certain embodiments, complementing sequences encoding proteins necessary to generate a transcribing, replicating HMPV nucleocapsid are provided by one or more helper viruses. Such helper viruses can be wild type or mutant. In one embodiment, the helper virus can be distinguished phenotypically from the virus encoded by the HMPV cDNA. For example, it may be desirable to provide monoclonal antibodies that react immunologically with the helper virus but not the virus

15   encoded by the HMPV cDNA. Such antibodies can be neutralizing antibodies. In some embodiments, the antibodies can be used in affinity chromatography to separate the helper virus from the recombinant virus. To aid the procurement of such antibodies, mutations can be introduced into the HMPV cDNA to provide antigenic diversity from the helper virus, such as in the HMPV glycoprotein genes.

20   Expression of the HMPV genome or antigenome and proteins from transfected plasmids can be achieved, for example, by each cDNA being under the control of a selected promoter (for example, for T7 RNA polymerase), which in turn is supplied by infection, transfection or transduction with a suitable expression system (for example, for the T7 RNA polymerase, such as a vaccinia virus MVA strain recombinant which expresses the T7 RNA polymerase, as described by

25   Wyatt et al., Virology 210:202-205, 1995). The viral proteins, and/or T7 RNA polymerase, can also be provided by transformed mammalian cells (see Buchholz et al., J. Virol. 73:251-259. 1999) or by transfection of preformed mRNA or protein.

A HMPV genome or antigenome can be constructed for use by, for example, assembling cloned cDNA segments, representing in aggregate the complete genome or antigenome, by PCR or

30   the like (described in, for example, U.S. Patent Nos. 4,683,195 and 4,683,202, and PCR Protocols: A Guide to Methods and Applications, Innis et al., eds., Academic Press, San Diego, 1990) of reverse-transcribed copies of HMPV mRNA or genome RNA. For example, a first construct can be generated which includes cDNAs containing the left hand end of the antigenome, spanning from an appropriate promoter (for example, T7 RNA polymerase promoter) and assembled in an appropriate

35   expression vector, such as a plasmid, cosmid, phage, or DNA virus vector. The vector can be modified by mutagenesis and/or insertion of a synthetic polylinker containing unique restriction sites designed to facilitate assembly. For ease of preparation the N, P, L and other desired HMPV proteins

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can be assembled in one or more separate vectors. The right hand end of the antigenome plasmid may contain additional sequences as desired, such as a flanking ribozyme and single or tandem T7 transcriptional terminators. The ribozyme can be hammerhead type, which would yield a 3' end containing a single nonviral nucleotide, or can be any of the other suitable ribozymes such as that of hepatitis delta virus (Perrotta *et al.*, Nature 350:434-436, 1991) that would yield a 3' end free of non-PIV nucleotides.

Alternative means to construct cDNA encoding the HMPV genome or antigenome include RT-PCR using different PCR conditions (for example, as described in Cheng *et al.*, Proc. Natl. Acad. Sci. USA 91:5695-5699, 1994) to reduce the number of subunit cDNA components to as few as one or two pieces. In other embodiments different promoters can be used (for example, T3, SPQ or different ribozymes, such as that of a hammerhead variety. Different DNA vectors (for example, cosmids) can be used for propagation to better accommodate the larger size genome or antigenome.

By "infectious clone" or "infectious cDNA" of HMPV is meant cDNA or its product, synthetic or otherwise, as well as RNA capable of being directly incorporated into infectious virions which can be transcribed into genomic or antigenomic HMPV RNA that can serve as a template to produce the genome of infectious HMPV viral or subviral particles. As noted above, defined mutations can be introduced into an infectious HMPV clone by a variety of conventional techniques (for example, site-directed mutagenesis) into a cDNA copy of the genome or antigenome. The use of genomic or antigenomic cDNA subfragments to assemble a complete genome or antigenome cDNA as described herein has the advantage that each region can be manipulated separately, where small cDNA constructs provide for better ease of manipulation than large cDNA constructs, and then readily assembled into a complete cDNA.

Isolated polynucleotides (for example, cDNA) encoding the HMPV genome or antigenome can be introduced into cells that can support a productive HMPV infection by transfection, electroporation, mechanical insertion, transduction or the like. Exemplary cells of use are HEp-2, FRhL-DBS2, LLC-MK2, MRC-5, baby hamster kidney (BHK), and Vero cells. Isolated polynucleotide sequences can be introduced into cultured cells by, for example, calcium phosphate-mediated transfection (Wigler *et al.*, Cell 14:725, 1978; Corsaro *et al.*, Somatic Cell Genetics 7:603, 1981; Graham *et al.*, Virology 52:456, 1973, electroporation (Neumann *et al.*, EMBO J. 1:841-845, 1982), DEAE-dextran mediated transfection (Ausubel *et al.*, (ed.) Current Protocols in Molecular Biology, John Wiley and Sons, Inc., NY, 1987), cationic lipid-mediated transfection (Hawley-Nelson *et al.*, Focus 15:73-79, 1993) or a commercially available transfection regent, for example, Lipofectamine-2000 (Invitrogen, Carlsbad, CA) or the like.

By providing infectious clones of HMPV, a wide range of alterations can be recombinantly produced within the HMPV genome (or antigenome), yielding defined mutations that specify desired phenotypic changes. The compositions and methods disclosed herein for producing recombinant HMPV permit ready detailed analysis and manipulation of HMPV molecular biology and pathogenic

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mechanisms using, for example, defined mutations to alter the function or expression of selected HMPV proteins. Using these methods and compositions, one can readily distinguish mutations responsible for desired phenotypic changes from silent incidental mutations, and select phenotype-specific mutations for incorporation into a recombinant HMPV genome or antigenome. In this

5   context, a variety of nucleotide insertions, deletions, substitutions, and rearrangements can be made in the HMPV genome or antigenome during or after construction of the cDNA. For example, specific desired nucleotide sequences can be synthesized and inserted at appropriate regions in the cDNA using convenient restriction enzyme sites. Alternatively, such techniques as site-specific mutagenesis, alanine scanning mutagenesis, PCR mutagenesis, or other such techniques well known

10   in the art can be used to introduce mutations into the cDNA.

Recombinant modifications of HMPV can be directed toward the production of improved candidate viruses, for example, to enhance viral attenuation and immunogenicity, to ablate epitopes associated with undesirable immunopathology, to accommodate antigenic drift, etc. To achieve these and other objectives, the compositions and methods disclosed herein allow for a wide variety of

15   modifications to be introduced into a HMPV genome or antigenome for incorporation into infectious, recombinant HMPV. For example, foreign genes or gene segments encoding antigenic determinants (for example, protective antigens or immunogenic epitopes) can be added within a HMPV clone to generate recombinant HMPVs capable of inducing immunity to both HMPV and another virus or pathogenic agent from which the antigenic determinants was/were derived. Alternatively, foreign

20   genes can be inserted, in whole or in part, encoding modulators of the immune system, such as cytokines, to enhance immunogenicity of a candidate virus. Other mutations that can be included within HMPV clones are, for example, substitution of heterologous genes or gene segments (for example, a gene segment encoding a cytoplasmic tail of a glycoprotein gene) with a counterpart gene or gene segment in a HMPV clone. Alternatively, the relative order of genes within a HMPV clone

25   can be changed, a HMPV genome promoter or other regulatory element can be replaced with its antigenome counterpart, or selected HMPV genes rendered non-functional (for example, by functional ablation involving introduction of a stop codon to prevent expression of the gene). In addition, the codon selection of genes such as those encoding the major protective antigens can be modified to improve the efficiency of translation. Other modifications in a HMPV clone can be made

30   to facilitate manipulations, such as the insertion of unique restriction sites in various non-coding or coding regions of the HMPV genome or antigenome. In addition, nontranslated gene sequences or intergenic regions can be shortened or removed to increase capacity for inserting foreign sequences.

As noted above, it is often desirable to adjust the phenotype of recombinant HMPVs for use by introducing additional mutations that attenuate the virus, affect the virulence of the virus, or

35   otherwise alter the phenotype of the recombinant virus. One of skill in the art can readily identify methods and procedures for mutagenizing, isolating and characterizing HMPV to obtain attenuated mutant strains (for example, temperature sensitive (*ts*), cold passaged (*cp*), cold-adapted (*ca*), small

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plaque (*sp*) and host-range restricted (*hr*) mutant strains) and for identifying the genetic changes that specify the attenuated phenotype (see, for example, Durbin *et al.*, Virology 235:323-332, 1997; U.S. Patent Application Serial No. 09/083,793, filed May 22, 1998; U.S. Patent Application Serial No. 09/458,813, filed December 10, 1999; U.S. Patent Application Serial No. 09/459,062, filed December
5   10, 1999; U.S. Provisional Application No. 60/047,575, filed May 23, 1997 (corresponding to International Publication No. WO 98/53078), and U.S. Provisional Application No. 60/059,385, filed September 19, 1997). Thus, methods are provided herein for determining replication, immunogenicity, genetic stability and immunogenic efficacy of biologically derived and recombinantly produced attenuated HMPVs in accepted model systems reasonably correlative of
10   human activity, including hamster or rodent and non-human primate model systems.

In additional embodiments, rHMPV candidates are constructed by introduction of nucleotide or amino acid point mutations that confer attenuation or other desired phenotypes. Such mutations can involve substitution of one or more nucleotides or amino acids at a given locus, or can involve small deletions in which one or more nucleotides or amino acids at a given locus are deleted. These
15   mutations are termed "point mutations" here to denote that each particular mutation is circumscribed. For example, a typical mutation can involve changing a single amino acid by substituting 1, 2 or 3 nucleotides in the corresponding codon. As another example, the three nucleotides might be deleted altogether, resulting in the deletion of a single amino acid in the encoded protein. Point mutations can be identified empirically, such as by systematically replacing charged amino acids in one or more of
20   the HMPV proteins with ones that are uncharged, for example, replacing aspartate, glutamate, lysine or arginine with alanine.

Alternatively, attenuating amino acid substitutions and other mutations can be devised using existing mononegavirus mutations as a guide, for example mutations in a different "biologically derived" HMPV, non-human pneumovirus, such as an APV, or non-HMPV virus, such as a RSV or
25   PIV. By "biologically derived" is meant any virus not produced by recombinant DNA methods. Thus, biologically derived HMPV include all naturally occurring HMPVs, including, for example, naturally occurring HMPV having a wild type genomic sequence and HMPV having allelic or mutant genomic variations from a reference wild type HMPV sequence, for example, HMPV having a mutation specifying an attenuated phenotype. Likewise, biologically derived HMPV include HMPV
30   mutants derived from a parental HMPV by, inter alia, artificial mutagenesis and selection procedures not involving direct recombinant DNA manipulation. Attenuating mutations in biologically derived HMPV and other nonsegmented negative stranded RNA viruses for incorporation within recombinant HMPV can occur naturally or can be introduced into wild type HMPV strains and thereafter identified and characterized by well known mutagenesis and analytic procedures. For example,
35   incompletely attenuated parental HMPV or other heterologous viral mutant strains can be produced by chemical mutagenesis during virus growth in cell cultures to which a chemical mutagen has been added, by selection of virus that has been subjected to passage at suboptimal temperatures in order to

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introduce growth restriction mutations, or by selection of a mutagenized virus that produces small plaques (sp) in cell culture. In addition, known mutations in existing mononegaviruses that have been produced by recombinant methods also can serve as a guide for devising desired mutations in HMPV. Such known mutations in heterologous viruses can be ones that had been deliberately

5   introduced by directed mutagenesis, or can be ones that arose spontaneously during the process of DNA manipulation and virus recovery and propagation, or can ones that arose in recombinantly derived viruses that were exposed subsequently to mutagenic agents or passaged in a manner designed to favor phenotypic changes, such as described above for generating mutations in biologically derived viruses.

10   In certain embodiments, the HMPV genome or antigenome is recombinantly modified to incorporate an attenuating mutation at an amino acid position corresponding to an amino acid position of an attenuating mutation identified in a heterologous, mutant nonsegmented negative stranded RNA virus. The virus can be either biologically derived or of recombinant origin. Based on routine sequence alignments and other analyses, mutations previously identified in a heterologous

15   HMPV or non-HMPV virus are mapped to a corresponding position in HMPV for "transfer" (that is, introduction of an identical, conservative or non-conservative mutation, potentially including a substitution, deletion or insertion, at a homologous or corresponding position identified by the alignment) into recombinant HMPV. In many cases, an alignment of the two sequences of interest is sufficient to identify the corresponding residue in HMPV, particularly if the percent amino acid

20   identity is substantial (for example, approximately 35-40% identity or greater) or high (for example, 70% identity or greater, such as at least about 80%, 90%, 95%, or 99% identity). In other cases, particularly when the amino acid relatedness is not substantial (for example, less than approximately 35% identity), additional heterologous related viruses can be included in the alignment in order to obtain reliably identify conserved residues that serve as markers to identify corresponding positions.

25   In one embodiment. the percentage of sequence identity associated with the terms "substantial" or "high" or "not high" are used herein in the context of comparison of heterologous viruses from different serotypes or taxonomic groups.

        A large assemblage of such candidate mutations are available for transfer into rHMPV of the current disclosure (see, for example, (Durbin *et al.*, Virology 235:323-332, 1997; Skiadopoulos *et al.*,

30   J. Virol. 72:1762-1768, 1998; Skiadopoulos *et al.*, J. Virol. 73:1374-1381, 1999; Skiadopoulos *et al.*, Virology 260:125-35, 1999; Durbin *et al.*, Virology 261:319-30, 1999; Newman *et al.*, Virus Genes 24:77-92, 2002; Feller *et al.*, Virology 276:190-201, 2000; U.S. Patent Application Serial No. 09/083,793, filed May 22, 1998; U.S. Patent Application Serial No. 09/458,813, filed December 10, 1999; U.S. Patent Application Serial No. 09/459,062, filed December 10, 1999; U.S. Provisional

35   Application No. 60/047,575, filed May 23, 1997 (corresponding to International Publication No. WO 98/53078); U.S. Provisional Application No. 60/059,385, filed September 19, 1997; U.S. Patent Application No. 10/302,547, filed November 21, 2002 and priority U.S. Provisional Application No.

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60/331,961, filed November 21, 2001; and U.S. Provisional Application No. 60/412,053, filed
September 18, 2002).

Within these embodiments, it is often desired to modify the recipient recombinant HMPV
genome or antigenome to encode an alteration at the subject site of mutation that corresponds
5   conservatively to the alteration identified in the heterologous mutant virus. For example, if an amino
acid substitution marks a site of mutation in the mutant virus compared to the corresponding wild
type sequence, then a similar substitution can be engineered at the corresponding residues in the
recombinant HMPV. In one example, the substitution will specify an identical or conservative amino
acid to the substitute residue present in the mutant viral protein (see below for a description of
10   conservative substations). However, it is also possible to alter the native amino acid residue at the
site of mutation non-conservatively with respect to the substitute residue in the mutant protein (for
example, by using any other amino acid to disrupt or impair the function of the wild type residue).
Negative stranded RNA viruses from which exemplary mutations are identified and transferred into a
recombinant HMPV of the current disclosure include heterologous strains of HMPV, other non-
15   HMPV pneumoviruses (for example, human RSV, bovine RSV, APV, pneumonia virus of mice
(PVM)), PIVs (for example, HPIV1, HPIV2, HPIV3, BPIV3, and MPIV1), Newcastle disease virus
(NDV), simian virus 5 (SV5), measles virus (MeV), rinderpest virus, canine distemper virus (CDV),
rabies virus (RaV), and vesicular stomatitis virus (VSV), among others.

As depicted in Figures 26A-26F, one or more corresponding sites of mutation that specify a
20   desired phenotypic change in a heterologous virus (for example, when indicated wild type residues at
the designated positions is/are altered, for example, by substitution) is identified by conventional
sequence alignment. The corresponding target sites for mutation (see, for example, boxed sites in
Figures 26A-26F) in HMPV is thereby mapped for transfer of the mutation into a recombinant
HMPV to yield attenuation or other desired phenotypic changes. More specifically, corresponding
25   amino acids between the compared heterologous mutant and HMPV sequences represent target sites
for identical or conservative transfer (for example, by recombinant engineering involving site-
directed mutagenesis of a HMPV antigenomic cDNA) of the subject mutation into a recombinant
HMPV. In some embodiments, attenuating and other desired mutations identified in one negative
stranded RNA virus are thereby targeted for transfer (for example, to be copied identically or
30   conservatively by substitution mutagenesis) into a corresponding position within the genome or
antigenome of a recombinant HMPV of the current disclosure. Related methods for rational design
of other recombinant mutant RNA viruses are described, for example, in International Application
No. PCT/US00/09695, filed April 12, 2000, published as WO 00/61737 on 10/19/00 corresponding to
U.S. National Phase application 09/958,292, filed on 01/08/02, and claiming priority to U.S.
35   Provisional Patent Application Serial No. 60/129,006, filed on April 13, 1999. Additional description
pertaining to this aspect of the current disclosure is provided in Newman *et al.*, Virus Genes 24:77-

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92, 2002; Feller *et al.*, Virology 10;276:190-201, 2000; Skiadopoulos *et al.*, Virology 260:125-35, 1999; and Durbin *et al.*, Virology 261:319-30, 1999.

In one exemplary embodiment, amino acid sequence alignments are made between the L gene of HMPV and the L genes of other mononegaviruses for which one or more attenuating substitutions in the L protein have been identified. This embodiment of the current disclosure is illustrated in FIGS. 26A-26F, which depict sequence alignments of various segments of the putative L protein of HMPV with corresponding segments of L proteins of a number of other mononegaviruses in which attenuating mutations have been mapped. As exemplified in FIGS. 26A-26F, various mutations identified in the L gene of a heterologous negative stranded RNA virus can be incorporated into recombinant HMPV to yield attenuation or other desired phenotypic changes. These figures provide exemplary, partial L gene sequence alignments between an HMPV wild type virus and the indicated viruses (including as examples, a different HMPV, RSV, HPIV1 HPIV3, and BPIV3). The examples of attenuating mutations include ones identified in viruses of biological or recombinant origin. The alignments are employed to identify regions containing known attenuating mutations in the heterologous virus.

FIGS. 26A-26F illustrate the identification and mapping of various exemplary attenuating mutations in the L gene for transfer into rHMPV (note that the numbers at the left indicate the positional reference number of the first residue in the partial sequence shown, and the numbers at the right indicate the positional reference number of the terminal residue in the partial sequence). FIG. 26A shows a Phenylalanine-521 to Leucine (F521L) substitution in the L protein specified by a mutation in the L gene of an RSV cold-passage temperature-sensitive (cpts) mutant cpts530 (Juhasz *et al.*, J. Virol. 71:5814-9, 1999). Also shown is an attenuating double mutation of Arginine-588-Alanine (R588A) and Aspartate-589-Alanine (D589A) that was originally developed in RSV (Tang *et al.*, Virology 302:207-16, 2002). FIG. 26B shows the positions of attenuating Tyrosine-942-Histidine (Y942H) and Leucine-992-Tyrosine (L992Y) mutations identified in the PIV3 cp45 vaccine candidate (Skiadopoulos *et al.* J. Virol. 72:31762-8, 1998; U.S. Patent Application No. 10/302,547, filed November 21, 2002 and priority U.S. Provisional Application No. 60/331,961, filed November 21, 2001; and U.S. Provisional Application No. 60/412,053, filed September 18, 2002). FIG. 26C shows the positions of the attenuating Isoleucine-1103-Valine (I1103V) mutation originally identified in BPIV3 (Haller *et al.*, Virology 288:342-50, 2001), the attenuating Threonine-1558-Isoleucine (T1558I) mutation identified in PIV3 cp45 (Skiadopoulos *et al.*, J. Virol. 72:31762-8, 1998), and the Cysteine-319-Tyrosine (C319Y) mutation identified the attenuated cpRSV vaccine candidate (Connors *et al.*, Virology 208:478-84, 1995). FIG. 26D shows the positions of the attenuating Glutamine-831-Leucine (Q831L) mutation identified in cpts248 RSV (Firestone *et al.*, Virology 225:419-22, 1996), the attenuating Methinone-1169-Valine (M1169V) mutation identified in the RSV mutant *cpts*530/109 (Juhasz *et al.*, J. Virol. 73:5176-80), the Aspartate-1183-Glutamate (D1183) mutation identified in the RSV mutant *cpts*248/404 (Firestone *et al.*, Virology 225:419-22,

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1996), and the combination of six point mutations that constitute the attenuating C9 cluster
characterized in recombinant RSV (Tang *et al.*, Virology 302:207-16, 2002): Aspartate-1187-Alanine
(D1187A), Lysine-1188-Alanine (K1188A), Arginine-1189-Alanine (R1189A), Glutamate-1190-
Alanine (E1190A), Glutamate-1208-Alanine (E1208A), and Arginine-1209-Alanine (R1209A). FIG.

5   26E shows the position of the attenuating Tyrosine-1321-Asparagine (Y1321N) mutation of RSV
cpts530/1030 (Whitehead *et al.*, J. Virol. 73:871-7, 1999), and the Histidine-1690-Tyrosine
(H1690Y) mutation of the attenuated cpRSV derivative (Connors *et al.*, Virology 208, 478-84, 1995).
FIG. 26F shows the positions of an attenuating Asparagine-43-Isoleucine (N43I) mutation identified
in the *cpts* RSV mutant 248/955, and the attenuating Threonine-1711-Isoleucine (T1711I) mutation

10   characterized in the chimeric virus rHPIV3-L$_B$, consisting of HPIV3 in which the L gene was
replaced by that of BPIV3 (Skiadopoulos *et al.*, J. Virol. 77:1141-8, 2003).

            The foregoing alignments show, for example, that the F521 residue in the RSV sequence is
exactly conserved among most of the other mononegaviruses examined (23 out of 24
paramyxoviruses and 1 out of 3 rhabdoviruses) and, in particular, is present in HMPV. It should be

15   noted that the amino acid position of the corresponding residue is not the same in each L protein,
reflecting differences in length as well as small deletions and insertions elsewhere in the various
individual molecules. Thus, the corresponding residue is F456 in HMPV. From this comparative
mapping analysis, the F456 residue in HMPV is identified as a target for either an identical,
conservative, or even non-conservative amino acid substitution (for example, substitution of the F456

20   residue by a leucine, or by a conservative or non-conservative amino acid as compared to leucine).
The same alignment (FIG. 26A) shows that the D589 assignment is also exactly conserved in each of
sequences. The R588 assignment is not conserved in each, but it is represented by the homologous
assignment of Lysine in HMPV. With respect to the attenuating Tyrosine-942-Histidine (Y942H)
and Leucine-992-Tyrosine (L992Y) mutations of PIV3 cp45, in both of these instances the exact

25   amino acid assignment was not conserved, but the presence of various highly-conserved residues
throughout the alignment show that these positions correspond (Figure 26B). Positional
correspondence in this context indicates that the aligned, corresponding target site for mutation
(whether it is occupied by an amino acid that is identical, conservative or divergent in structural
character compared the corresponding residue in the wild type (for example, a non-attenuated parent)

30   sequence of the heterologous virus) will often yield an attenuation or other desired phenotype in a
rHMPV upon mutation from the HMPV wild type sequence to a different residue (that is, to a residue
identity that is the same, conservative, or even distinct structurally in comparison to the
corresponding residue in the heterologous mutant).

            In addition to the foregoing L gene mutations, two temperature sensitive (ts) mutations that

35   were reported in the P protein of recombinant RSV, namely Glycine-172-Serine and Glutamate-176-
Glycine (Lu *et al.*, J. Virol. 76:2871-80, 2002), can be transferred to yield a predicted attenuation
phenotype in a rHMPV P, by altering the corresponding one or both of the HMPV residues Glycine-

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213 and Glutamate-217, respectively. Likewise, a mutation in the N protein of the attenuated derivative cpRSV, specifying a Valine-267-Isoleucine substitution (Connors *et al.* Virology 208:478-84, 1996), maps to Serine-268 in HMPV, whereby an Ile or other, conservative or non-conservative, substitution of this residue in a rHMPV can yield useful candidates for identification of additional

5   attenuated derivatives. In the same manner, two mutations in the F protein of cpRSV, namely Glutamate-218-Alanine and Threonine-523-Isoleucine (Connors *et al.* Virology 208:478-84, 1996), correspond to Lysine-188 and Threonine-489, respectively, in the HMPV F protein, which therefore represent target sites for introduction of an attenuating amino acid substitution in rHMPV.

In other embodiments, mutations in a cis-acting regulatory sequence or other non-coding

10   sequence are identified in a heterologous virus (including biologically or otherwise derived mutant HMPV of the same or different strain, subtype, species, RSV, PIV, APV, etc.) and incorporated in a rHMPV of the current disclosure. For example, the identification of the GS and GE motifs identifies these as targets for mutagenesis. The short, circumscribed nature of these signals is amenable to a systematic evaluation of the effects of mutations at each position. Mutations in RNA signals such as

15   these can be readily assayed in a mini-replicon system as well as in complete virus.

FIG. 28 shows an example of transferring a mutation that involves a cis-acting RNA signal. A potent attenuating point mutation identified for the cpts 248/404 derivative of RSV involves a point mutation in the GS signal of the M2 gene: specifically, the T residue (positive sense) at position nine was changed to C (see, for example, Whitehead *et al.*, Virology 247:232-9, 1998). In the proposed

20   GS signal of HMPV, a T residue at position nine is conserved in each of the proposed HMPV genes (FIG. 28). This substitution can be made in any of the HMPV genes.

In accordance with the foregoing description, a large panel of desired mutations identified in heterologous viruses can be "transferred" into a rHMPV, even in cases of low sequence relatedness. In these embodiments, there is not a strict requirement that the corresponding wild type or final

25   mutant amino acid assignment be identical to that of the heterologous virus. Also, rather than an amino acid substitution, it is possible to delete one or several amino acids or, alternatively, insert one or several amino acids. In designing substitution mutations for HMPV, a useful strategy is to evaluate all possible alternative amino acid assignments at this position. This is a useful method for evaluating the full range of possible attenuation phenotypes involving this position, and can produce

30   mutants exhibiting a range of temperature sensitivity and attenuation (see, for example, U.S. Patent Application No. 10/302,547, filed November 21, 2002 and priority U.S. Provisional Application No. 60/331,961, filed November 21, 2001; and U.S. Provisional Application No. 60/412,053, filed September 18, 2002). In addition, the choice of codon for substitution can be made to involve the greatest number of nucleotide differences relative to both the wild type and to any possible alternative

35   amino acid assignments that are found to not yield attenuated phenotypes. This has the effect of reducing the probability of reversion or mutation to lose the attenuated phenotype. Specifically, it is estimated that the frequency of reversion at any single nucleotide position is 10^{-4} to 10^{-5}, and hence

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the frequency of reversion/mutation at two nucleotide positions is 10^{-8} to 10^{-10}, and the frequency of reversion/mutation involving three nucleotides is 10^{-12} to 10^{-15}. Thus, point mutations involving multiple nucleotide substitutions can greatly increase genetic and phenotypic stability. Also, the mutation can be designed to involve deletion of a single amino acid or more than one amino acid,

5   which can reduce the possibility of reversion.

It should be noted that the mutations illustrated in FIGS. 26A-26F are only exemplary by virtue that the depicted mapping exercise was limited to examples involving amino acid changes in the L protein, whereas the same strategy can be applied in accordance with the tools and description provided herein to transfer attenuating mutations involving any viral protein. For example, two *ts*

10 mutations that were developed in the P protein of recombinant RSV, namely Glycine-172-Serine and Glutamate-176-Glycine (Lu *et al.*, J. Virol. 76:2871-80, 2002), correspond to HMPV P residues Glycine-213 and Glutamate-217, respectively. A mutation in the N protein of the attenuated derivative *cp*RSV, namely Valine-267-Isoleucine (Connors *et al.* Virology 208:478-84, 1996), corresponds to Serine-268 in HMPV. Also, two mutations in the F protein of *cp*RSV, namely

15 Glutamate-218-Alanine and Threonine-523-Isoleucine (Connors *et al.* Virology 208:478-84, 1996), correspond to Lysine-188 and Threonine-489, respectively, in the HMPV F protein. Thus, any known mutation having a desirable phenotypic property present in any nucleotide or amino acid sequence is a useful candidate for incorporation in rHMPV by mutation of corresponding residues in a rHMPV RNA or protein employing the novel tools and methods of the current disclosure.

20 Another method for devising attenuating or otherwise desirable amino acid substitution mutations involve "charge-to-alanine" or "alanine scanning" mutagenesis where, for example, one or more charged amino acid residues are each changed to alanine residues (Wertman *et al.*, Genetics 132:337-50, 1992; Hassett and Condit, Proc. Natl. Acad. Sci. USA 91:4554-58, 1994; Tang *et al.*, Virology 302:207-16, 2002. A typical target is a pair of residues, but residues can be changed singly

25 or in larger combinations. It is often useful to systematically change ("scan") residues across an encoded protein. Any protein can be a target, although the L protein is the most common locus of mutations in attenuated mononegaviruses. Alternatively, one advantage of this strategy is that one can target proteins that are not represented in available mutants. This strategy can be readily addressed with the methods disclosed herein. In particular, the minireplicon system offers a method for

30 screening mutations in the N, P, L and M2-1 proteins. In that strategy, mutations are introduced into the appropriate support plasmid, such as the one encoding the L protein, and evaluated for the ability to direct the replication and transcription of a minigenome containing a reporter gene. For example, the assay can be done in replica at 32°C and 37°C (or any temperature in the range of 35°C -41°C) to identify ts mutations that operate at the lower, permissive temperature and are inhibited at the higher

35 temperature. This offers a more rapid and technically simple method for performing an initial screening, after which appropriate mutations can be introduced into complete recombinant virus.

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Thus, the methods disclosed herein can be used to develop mutations that confer improved properties to HMPV for the purposes of developing an immunogenic composition or for other purposes, such as purifying viral proteins. Mutations can be optimized, such as by evaluating all possible amino acid or nucleotide assignments at a given position, or by making amino acid changes involve codon choices that will be less likely to revert to wild type or to an alternative assignment that yields a wild type phenotype. Other changes can be made that fundamentally alter the viral backbone, such as changes in gene order or movement of major protective antigen genes to a promoter-proximal position. Importantly, these various modifications can be introduced into rHMPV in combination to develop viruses for use in immunogenic compositions or for other purposes. The methods described herein allows for the systematic modification of viruses as necessary, such as the introduction of additional attenuating mutations into a recombinant virus in response to clinical studies.

As noted above, production of a sufficiently attenuated biologically derived HMPV or other viral mutant can be accomplished by several known methods. One such procedure involves subjecting a partially attenuated virus to passage in cell culture at progressively lower, attenuating temperatures. For example, partially attenuated mutants are produced by passage in cell cultures at suboptimal temperatures. Thus, a cold-adapted (*ca*) mutant or other partially attenuated HMPV strain is adapted to efficient growth at a lower temperature by passage in culture. This selection of mutant HMPV during cold-passage substantially reduces any residual virulence in the derivative strains as compared to the partially attenuated parent. Alternatively, specific mutations can be introduced into biologically derived HMPV by subjecting a partially attenuated parent virus to chemical mutagenesis, for example, to introduce ts mutations or, in the case of viruses which are already *ts*, additional ts mutations sufficient to confer increased attenuation and/or stability of the ts phenotype of the attenuated derivative. Means for the introduction of *ts* mutations into HMPV include replication of the virus in the presence of a mutagen such as 5-fluorouridine according to generally known procedures. Other chemical mutagens can also be used. Attenuation can result from a *ts* mutation in almost any HMPV gene, although a particularly amenable target for this purpose has been found to be the polymerase (L) gene. The level of temperature sensitivity of replication in exemplary attenuated HMPV can be determined by comparing its replication at a permissive temperature with that at several restrictive temperatures. The lowest temperature at which the replication of the virus is reduced 100-fold or more in comparison with its replication at the permissive temperature is termed the "shutoff temperature." In experimental animals and humans, both the replication and virulence of HMPV correlate with the mutant's shutoff temperature.

From biologically and recombinantly derived HMPV and other nonsegmented negative stranded RNA viruses, a large "menu" of attenuating mutations is identifiable by the teachings herein, each of which can be combined with any other mutations for adjusting the level of attenuation, immunogenicity and genetic stability in recombinant HMPV. In this context, many recombinant

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HMPV candidates will include one or more, and preferably two or more, mutations from a biologically derived HMPV or other heterologous viral mutant, for example, any one or combination of mutations identified in APV, HPIV, BPIV3, and/or RSV. Preferred recombinant HMPVs can incorporate a plurality of mutations thus identified. Often, these mutations are stabilized against reversion in recombinant HMPV by multiple nucleotide substitutions in a codon specifying each mutation.

Mutations compiled into a "menu" as described above are introduced as desired, singly or in combination, to adjust recombinant HMPV to an appropriate level of attenuation, immunogenicity, genetic resistance to reversion from an attenuated phenotype, etc. In accordance with the foregoing description, the ability to produce infectious recombinant HMPV from cDNA permits introduction of specific engineered changes within the recombinant HMPV. In particular, infectious, recombinant HMPVs can be employed for further identification of specific mutations in biologically derived, attenuated HMPV strains, for example mutations that specify *ts*, *ca*, *att* and other phenotypes. Desired mutations identified by this and other methods are introduced into recombinant HMPV candidate strains. The capability of producing virus from cDNA allows for routine incorporation of these mutations, individually or in various selected combinations, into a full-length cDNA clone, where after the phenotypes of rescued recombinant viruses containing the introduced mutations can be readily determined.

By identifying and incorporating specific mutations associated with desired phenotypes, for example, a *ca* or *ts* phenotype, into infectious recombinant HMPV, additional site-specific modifications at, or within close proximity to, the identified mutation are identified. Whereas most attenuating mutations produced in biologically derived HMPVs are single nucleotide changes, other "site specific" mutations can also be incorporated by recombinant techniques into a recombinant HMPV. As used herein, site-specific mutations include insertions, substitutions, deletions or rearrangements of from about 1 to about 3, up to about 5-15 or more altered nucleotides (for example, altered from a wild type HMPV sequence, from a sequence of a selected mutant HMPV strain, or from a parent recombinant HMPV clone subjected to mutagenesis). Such site-specific mutations can be incorporated at, or within the region of, a selected, biologically derived point mutation. Alternatively, the mutations can be introduced in various other contexts within a recombinant HMPV clone, for example at or near a cis-acting regulatory sequence or nucleotide sequence encoding a protein active site, binding site, immunogenic epitope, etc.

Site-specific recombinant HMPV mutants typically retain a desired attenuating phenotype, but can additionally exhibit altered phenotypic characteristics unrelated to attenuation, for example, enhanced or broadened immunogenicity, and/or improved growth. Further examples of desired, site-specific mutants include recombinant HMPV mutants engineered to incorporate additional, stabilizing nucleotide mutations in a codon specifying an attenuating point mutation. Where possible, two or more nucleotide substitutions are introduced at codons that specify attenuating amino acid

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changes in a parent mutant or recombinant HMPV clone, yielding a recombinant HMPV with greater genetic resistance to reversion from an attenuated phenotype. In other embodiments, site-specific nucleotide substitutions, additions, deletions or rearrangements are introduced upstream or downstream, e. g., from about 1 to about 3, about 5-10 and up to 15 nucleotides or more 5' or 3',
5   relative to a targeted nucleotide position, for example, to construct or ablate an existing cis-acting regulatory element.

In addition to single and multiple point mutations and site-specific mutations, changes to the recombinant HMPV include deletions, insertions, substitutions or rearrangements of one or more genes or genome segments to increase, decrease, ablate or otherwise alter gene expression.
10   Expression of one or more non-essential genes can be reduced or ablated by modifying the recombinant HMPV genome or antigenome, for example, to incorporate a mutation that alters the coding assignment of an initiation codon or mutations that introduce one or one or more stop codons. Alternatively, one or more non-essential genes or genome segments can be deleted in whole or in part to render the corresponding proteins partially or entirely non-functional or to disrupt protein
15   expression altogether. Exemplary recombinant HMPV within these aspects of the current disclosure are provided herein that exhibit partial or complete deletions of the G, SH and/or M2-2 ORFs, including an exemplary mutant that has both the G and SH genes deleted. These and other recombinants can be engineered and selected to possess highly desirable phenotypic characteristics for development of immunogenic compositions. For example, these modifications can specify one or
20   more desired phenotypic changes including (i) altered growth properties in cell culture, (ii) attenuation in the upper and/or lower respiratory tract of mammals, (iii) a change in viral plaque size, (iv) a change in cytopathic effect, (v) a change in immunogenicity, and (vi) a change in RNA replication and gene expression.

Thus a recombinant HMPV can incorporate one or more partial or complete gene deletions,
25   knock out mutations, or mutations that simply reduce or increase expression of an HMPV gene. This can be achieved, for example, by introducing a frame shift mutation or termination codon within a selected coding sequence, altering translational start sites, changing the position of a gene or introducing an upstream start codon to alter its rate of expression, changing GS and/or GE transcription signals to alter phenotype, or modifying an RNA editing site (for example, growth,
30   temperature restrictions on transcription, etc.). In several examples, recombinant HMPVs are provided in which expression of one or more genes, for example, a SH, G, or M2-2 ORF, is ablated at the translational or transcriptional level without deletion of the gene or of a segment thereof, by, for example, introducing multiple translational termination codons into a translational ORF, altering an initiation codon, or modifying an editing site. In one embodiment, expression of the M2-2 ORF is
35   ablated by removal of both potential translational start codons, in combination with partial deletion of the M2-2 ORF. These and other forms of knock-out virus will often exhibit reduced growth rates and small plaque sizes in tissue culture. Thus, additional novel types of attenuating mutations are

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provided herein which ablate or alter expression of a viral gene. In this context, knock-out virus phenotypes produced without deletion of a gene or genome segment can be alternatively produced by deletion mutagenesis, as described, to effectively preclude correcting mutations that may restore synthesis of a target protein. Other gene knock-outs can be made using alternate designs and

5   methods that are well known in the art (as described, for example, in (Kretschmer *et al.*, Virology 216:309-316, 1996; Radecke *et al.*, Virology 217:418-421, 1996; Kato *et al.*, EMBO J. 16:578-587, 1987; and Schneider *et al.*, Virology 277:314-322, 1996).

Nucleotide modifications that may be introduced into recombinant HMPV constructs can alter small numbers of bases (for example, from 15-30 bases, up to 35-50 bases or more), large blocks

10   of nucleotides (for example, about 50-100, about 100-300, about 300-500, about 500-1,000 bases), or nearly complete or complete genes (for example, about 1,000-1,500 nucleotides, about 1,500-2,500 nucleotides, about 2,500-5,000, nucleotides, about 5,000-6,0000 nucleotides or more) in the vector genome or antigenome or heterologous, donor gene or genome segment, depending upon the nature of the change (that is, a small number of bases may be changed to insert or ablate an immunogenic

15   epitope or change a small genome segment, whereas large blocks of bases are involved when genes or large genome segments are added, substituted, deleted or rearranged).

In related aspects, the current disclosure provides for supplementation of mutations adopted into a recombinant HMPV clone from biologically derived HMPV, for example, *ca* and *ts* mutations, with additional types of mutations involving the same or different genes in a further modified

20   recombinant HMPV. Each of the HMPV genes can be selectively altered in terms of expression levels, or can be added, deleted, substituted or rearranged, in whole or in part, alone or in combination with other desired modifications, to yield a recombinant HMPV exhibiting novel characteristics. Thus, in addition to or in combination with attenuating mutations adopted from biologically derived HMPV and/or non-HMPV mutants, the current disclosure also provides a range

25   of additional methods for attenuating or otherwise modifying the phenotype of a recombinant HMPV based on recombinant engineering of infectious HMPV clones. A variety of alterations can be produced in an isolated polynucleotide sequence encoding a targeted gene or genome segment, including a donor or recipient gene or genome segment in a recombinant HMPV genome or antigenome for incorporation into partially attenuated, infectious clones. More specifically, to

30   achieve desired structural and phenotypic changes in recombinant HMPV, the current disclosure allows for introduction of modifications which delete, substitute, introduce, or rearrange a selected nucleotide or nucleotide sequence from a parent genome or antigenome, as well as mutations which delete, substitute, introduce or rearrange whole genes or genome segments, within a recombinant HMPV.

35   Thus provided are modifications in recombinant HMPV which simply alter or ablate expression of a selected gene, for example, by introducing a termination codon within a selected HMPV coding sequence or altering its translational start site or RNA editing site, changing the

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position of a HMPV gene relative to an operably linked promoter, introducing an upstream start codon to alter rates of expression, modifying (for example, by changing position, altering an existing sequence, or substituting an existing sequence with a heterologous sequence) GS and/or GE transcription signals to alter phenotype (for example, growth, temperature restrictions on

5   transcription, etc.), and various other deletions, substitutions, additions and rearrangements that specify quantitative or qualitative changes in viral replication, transcription of selected genes, or translation of selected proteins. In this context, any HMPV gene or genome segment which is not essential for growth can be ablated or otherwise modified in a recombinant HMPV to yield desired effects on virulence, pathogenesis, immunogenicity and other phenotypic characters. In addition to

10   coding sequences, noncoding, leader, trailer and intergenic regions can be similarly deleted, substituted or modified and their phenotypic effects readily analyzed, for example, by the use of minireplicons, and the recombinant HMPV described herein.

In addition to these changes, the order of genes in a recombinant HMPV construct can be changed, a HMPV genome promoter replaced with its antigenome counterpart or vice versa, portions

15   of genes removed or substituted, and even entire genes deleted. Different or additional modifications in the sequence can be made to facilitate manipulations, such as the insertion of unique restriction sites in various intergenic regions or elsewhere. Nontranslated gene sequences can be removed to increase capacity for inserting foreign sequences.

Other mutations for incorporation into recombinant HMPV constructs of the current

20   disclosure include mutations directed toward cis-acting signals, which can be readily identified, for example, by mutational analysis of HMPV minigenomes. For example, insertional and deletional analysis of the leader, trailer and/or flanking sequences identifies viral promoters and transcription signals and provides a series of mutations associated with varying degrees of reduction of RNA replication or transcription. Saturation mutagenesis (whereby each position in turn is modified to

25   each of the nucleotide alternatives) of these cis-acting signals also can be employed to identify many mutations that affect RNA replication or transcription. Any of these mutations can be inserted into a chimeric HMPV antigenome or genome as described herein. Evaluation and manipulation of trans-acting proteins and cis-acting RNA sequences using the complete antigenome cDNA is assisted by the use of HMPV minigenomes as described in the above-incorporated references.

30   Additional mutations within recombinant HMPVs can also include replacement of the 3' end of genome with its counterpart from antigenome or vice versa, which is associated with changes in RNA replication and transcription. In one exemplary embodiment, the level of expression of specific HMPV proteins, such as the protective HN and/or F antigens, can be increased by substituting the natural sequences with ones which have been made synthetically and designed to be consistent with

35   efficient translation. In this context, it has been shown that codon usage can be a major factor in the level of translation of mammalian viral proteins (Haas *et al.*, Current Biol. 6:315-324, 1996). Optimization by recombinant methods of the codon usage of the mRNAs encoding one or more

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immunogenic glycoproteins of recombinant HMPV will provide improved expression for these genes.

In another exemplary embodiment, a sequence surrounding a translational start site (preferably including a nucleotide in the -3 position relative to the AUG start site) of a selected HMPV gene or donor gene incorporated in an HMPV vector is modified, alone or in combination with introduction of an upstream start codon, to modulate gene expression by specifying up- or down-regulation of translation. Alternatively, or in combination with other recombinant modifications disclosed herein, gene expression of a recombinant HMPV can be modulated by altering a transcriptional GS or GE signal of any selected genes of the virus. In alternative embodiments, levels of gene expression in a recombinant HMPV candidate are modified at the level of transcription. In one aspect, the position of a selected gene in the HMPV gene map can be changed to a more promoter-proximal or promoter-distal position, whereby the gene will be expressed more or less efficiently, respectively. According to this aspect, modulation of expression for specific genes can be achieved yielding reductions or increases of gene expression from two-fold, more typically four-fold, up to ten-fold or more compared to wild type levels often attended by a commensurate decrease in expression levels for reciprocally, positionally substituted genes. These and other transpositioning changes yield novel recombinant HMPVs having attenuated phenotypes, for example due to decreased expression of selected viral proteins involved in RNA replication, or having other desirable properties such as increased antigen expression.

In the latter context, any one or combination of antigenic glycoproteins, prospectively including F, G, and SH, or antigenic determinants thereof, can be produced at elevated or decreased levels (or with otherwise enhanced immunogenic activity) by, for example, changing the promoter-relative position of the subject gene or genome segment. For example, the wild type gene order of the SH, G and F ORFs of HMPV are located at gene positions 6, 7 and 4, respectively. Recombinantly altering the promoter proximity of one or more of these genes will modulated its expression, and/or expression of normally "upstream" and "downstream" genes. The change in gene expression due to the relocation of genes or to the addition of a second copy can have other desirable effects, such as increased or decreased growth *in vitro* or *in vivo*. In exemplary embodiments, placement of putative protective antigen genes such as SH, G, and F more proximal to the promoter, either singly, as a pair, or as a triplet, will result in more efficient expression. As described herein, exemplary embodiments of "promoter-shifted" HMPV are provided wherein the position of the SH-G gene pair was altered, for example by moving the pair from its wild type gene order position following the M2 gene to a position preceding the F gene. This resulted in a gene order in that region of the genome that mimics that of RSV. In another exemplary embodiment, the SH-G pair was moved to be upstream of the F gene, and a second copy of the pair was inserted, resulting in multiple copies of the SH and G prospective antigens. In yet additional embodiments, the prospective antigens comprising one or more of the G and F proteins are modified in their expression by

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promoter-relative shifting of these genes (having wild type gene order positions at the 7th and 4th gene positions, respectively in the HMPV genome map) to more promoter-proximal positions.

In other embodiments, recombinant HMPVs useful in immunogenic compositions can be conveniently modified to accommodate antigenic drift in circulating virus. In this context, the
5   Examples below provide the complete sequence for an HMPV genome, and also provide a detailed comparison of complete genome sequences for HMPV isolates representing two distinct antigenic subgroups of HMPV, that are generally comparable in sequence divergence to the antigenic subgroups of HRSV. Identification and characterization of these distinct antigenic subgroups, including detailed mapping of their prospective antigenic glycoprotein gene structure, provides for
10   construction of novel recombinant HMPVs for use in immunogenic compositions to elicit an immune response against one or more HMPV strains, including in bivalent immunogenic composition to immunize against multiple HMPV strains and/or accommodate antigenic drift. Typically the recombinant HMPV will have a modification in one or more of the SH, G, and/or F glycoproteins. For example, an entire SH, G, and/or F gene, or a genome segment encoding a particular antigenic
15   determinant (for example, immunogenic region or epitopes) thereof), from one HMPV strain or group can be incorporated into a recombinant HMPV genome or antigenome cDNA by replacement of a corresponding region in a recipient clone of a different HMPV strain or group, or by adding one or more copies of the gene, such that multiple antigenic forms are represented in the recombinant virus. Progeny virus produced from the modified recombinant HMPV can then be used in immunization
20   protocols against multiple, and emerging, HMPV strains.

In certain aspects of the current disclosure, replacement of a HMPV coding sequence or non-coding sequence (for example, a promoter, GE, GS, intergenic or other cis-acting element) with a heterologous (for example, non-HMPV) counterpart yields chimeric HMPV having a variety of possible attenuating and other phenotypic effects. For example, host range and other desired effects
25   can be engineered by importing an APV or other non-HMPV virus (for example, RSV, SV5, SV41, NDV, PIV) protein, protein domain, gene or genome segment into a recombinant HMPV "background" genome or antigenome, wherein the heterologous or "donor" gene or genome segment does not function efficiently in a human cell, for example, from incompatibility of the heterologous sequence or protein with a biologically interactive HMPV sequence or protein (that is, a sequence or
30   protein that ordinarily cooperates with the substituted sequence or protein for viral transcription, translation, assembly, etc.) or, more typically in a host range restriction, with a cellular protein or some other aspect of the cellular milieu which is different between the permissive and less permissive host. In exemplary embodiments, APV, pneumovirus of mice, or RSV sequences are selected for introduction into HMPV based on aspects of HMPV structure and function described herein.
35   Methods are provided herein for attenuating recombinant HMPV candidates based on the construction of chimeras between HMPV and a non-human pneumovirus or other negative stranded RNA virus, for example APV, RSV, HPIV, MPIV1 (Sendai virus), BPIV3, SV5, SV41, and/or NDV

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(for example, as disclosed in U.S. Patent Application Serial No. 09/586,479, filed June 1, 2000 by Schmidt *et al.* (corresponding to PCT Publication WO 01/04320); Schmidt *et al.*, J. Virol. 74:8922-9, 2000). In exemplary embodiments, the recombinant HMPV genome or antigenome is combined with a heterologous gene or genome segment, such as an N, P, M, or L, ORF derived from a non-HMPV

5   paramyxovirus.

Paramyxoviruses are enveloped RNA viruses that include two subfamilies (*Paramyxovirinae* and *Pneumovirinae*). Subfamily *Paramyxovirinae* has five different genera, including *Respirovirus* (containing parainfluenza viruses 1 and 3), *Rubulavirus* (containing parainfluenza viruses 2 and 4 and mumps), and *Morbillivirus* (containing measles and canine

10 distemper viruses). Subfamily *Pneumovirinae* has two genera, namely *Pneumovirus* (containing RSV) and *Metapneumovirus* (containing HMPV). Paramyxoviruses encoded 6-11 proteins. Proteins common to all paramyxoviruses include the nucleocapsid N protein that associates tightly with the RNA genome to form the nucleocapsid, the phosphoprotein P and large L polymerase protein that also associate with the nucleocapsid, the matrix M protein that associates with the inner face of the

15 viral envelope, the transmembrane fusion F glycoprotein that mediates penetration, and the attachment protein that, depending on the virus, is called glycoprotein G, or the hemagglutinin HA or the hemagglutinin-neuraminidase HN. Other proteins are found in some viruses but not others, such as the nonstructural NS1 and NS2 proteins of RSV, the M2-1 and M2-2 proteins found in RSV and, as disclosed here, in HMPV, the SH protein that is found in mumps, simian virus 5, RSV and HMPV,

20 and the C, D and V proteins found in certain parainfluenza viruses. The sequences of non-HMPV HN, M, N, P, L, F, G, and SH proteins, and nucleic acids encoding these proteins are known in the art (see, Lamb, RA and Kolakofsky, D. pp 1305-1340 in Fields Virology, 4th addition, Knipe and Howley (eds) Lippincott Williams and Wilkins, Philadelphia, 2001, Chanock, Murphy and Collins, pp 1341-1379, ibid, Collins, Chanock and Murphy, pp 1443-1485, ibid.).

25 Chimeric HMPV are therefore provided herein that include a partial or complete "background" HMPV genome or antigenome derived from or patterned after HMPV combined with one or more heterologous genes or genome segments of a non- HMPV virus to form the chimeric HMPV genome or antigenome. In one embodiment, chimeric HMPV of this type incorporate a partial or complete HMPV background genome or antigenome combined with one or more

30 heterologous genes or genome segments. The partial or complete background genome or antigenome typically acts as a recipient backbone into which the heterologous genes or genome segments of the counterpart, non- HMPV virus are incorporated. Heterologous genes or genome segments from the counterpart virus represent "donor" genes or polynucleotides that are combined with, or substituted within, the background genome or antigenome to yield a chimeric HMPV that exhibits novel

35 phenotypic characteristics compared to one or both of the contributing viruses. For example, addition or substitution of heterologous genes or genome segments within a selected recipient HMPV strain may result in an increase or decrease in attenuation, growth changes, altered immunogenicity,

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or other desired phenotypic changes as compared with a corresponding phenotypes of the unmodified recipient and/or donor (see, for example, U.S. Patent Application Serial No. 09/586,479, filed June 1, 2000 by Schmidt *et al.*; Schmidt *et al.*, J. Virol. 74:8922-9, 2000).

5   Genes and genome segments that can be selected for use as heterologous substitutions or additions within chimeric HMPV include genes or genome segments encoding a HMPV N, P, M, F, M2, SH, G and/or L proteins or portions thereof. In addition, genes and genome segments encoding proteins found in other viruses, (for example, an SH protein as found in mumps, RSV, and SV5 viruses), may be incorporated within additional chimeric HMPV recombinants of the current disclosure. Regulatory regions from heterologous viruses, for example the extragenic 3' leader or 5'
10 trailer regions, and GS, GE, intergenic regions, or 3' or 5' non-coding regions, are also useful as substitutions or additions within recombinant HMPV. In exemplary aspects, chimeric HMPV bearing one or more non-HMPV genes or genome segments exhibit a high degree of host range restriction, for example, in the respiratory tract of mammalian models of human HMPV infection such as hamsters and non-human primates. In more detailed embodiments HMPV is attenuated by
15 the addition or substitution of one or more APV or RSV genes or genome segments selected from N, P, M, F, M2, SH, G and/or L genes and genome segments to a partial or complete HMPV background genome or antigenome.

     In one embodiment, the degree of host range restriction exhibited by chimeric HMPV for use within immunogenic compositions of the current disclosure is comparable to the degree of host
20 range restriction exhibited by the respective non-HMPV "donor" strain. For example, the restriction should have a true host range phenotype, that is, it should be specific to the host in question and should not restrict replication *in vitro* in a suitable cell line. In addition, chimeric HMPV bearing one or more heterologous genes or genome segments elicit a desired immunogenic response in hosts susceptible to HMPV infection. Thus, the current disclosure provides a new basis for attenuating a
25 live HMPV virus vector for developing immunogenic compositions against HMPV and other pathogens based on host range effects.

     In combination with the host range phenotypic effects provided in the chimeric HMPV, it is often desirable to adjust the attenuation phenotype by introducing additional mutations that increase or decrease attenuation of the chimeric virus. Thus, in additional embodiments, attenuated, chimeric
30 HMPV are produced in which the chimeric genome or antigenome is further modified by introducing one or more attenuating mutations specifying an attenuating phenotype in the resultant virus or subviral particle. These can include mutations generated de novo and tested for attenuating effects according to a rational design mutagenesis strategy. Alternatively, the attenuating mutations may be identified in existing biologically derived mutant HMPV or non-HMPVs and thereafter incorporated
35 into a chimeric HMPV of the current disclosure. Exemplary mutations specify lesions in RNA regulatory sequences or in encoded proteins.

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In certain chimeric HMPV, attenuation marked by replication in the lower and/or upper respiratory tract in an accepted animal model that is reasonably correlated with HMPV replication and immunogenic activity in humans (for example, hamsters, rhesus monkeys or chimpanzees), is reduced by at least about two-fold, more often about 5-fold, 10-fold, or 20-fold, and preferably 50-

5   100-fold and up to 1,000-fold or greater overall (for example, as measured between 3-8 days following infection) compared to growth of the corresponding wild type or mutant parental strains.

Within the methods disclosed herein, additional genes or genome segments can be inserted into or proximate to a recombinant or chimeric HMPV genome or antigenome. For example, various supernumerary heterologous genes or genome segments can be inserted at any of a variety of sites

10   within the recombinant genome or antigenome, for example at a position 3' to N, between the N/P, P/M, M/F, F/M2, M2/SH, SH/G, and/or G/L genes, or at another non-coding region of the HMPV vector genome or antigenome (see FIG. 1). The inserted genes can be under common control with recipient genes, or can be under the control of an independent set of transcription signals. Genes of interest in this context include genes encoding cytokines, for example, an interleukin (IL-2 through

15   IL-18, for example, interleukin 2 (IL-2), interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 6 (IL-6), interleukin 18 (IL-18), tumor necrosis factor alpha (TNFα), interferon gamma (IFNγ), or granulocyte-macrophage colony stimulating factor (GM-CSF) (see, for example, U.S. Application No. 09/614,285, filed July 12, 2000 and priority U.S. Provisional Application Serial No. 60/143,425 filed July 13, 1999). Coexpression of these additional proteins provides the ability to modify and

20   improve immune responses against recombinant HMPV quantitatively and/or qualitatively.

In additional embodiments, insertion of heterologous nucleotide sequences into recombinant HMPV candidates are employed separately to modulate the level of attenuation of candidate recombinants, for example, for the upper respiratory tract. Thus, it is possible to insert nucleotide sequences into a recombinant HMPV that both direct the expression of a foreign protein and that

25   attenuate the virus in an animal host, or to use nucleotide insertions separately to attenuate candidate viruses. To define some of the rules that govern the effect of gene insertion on attenuation, gene units of varying lengths are inserted into a wild type HMPV backbone and the effects of gene unit length on attenuation examined. Novel gene unit insertions are contemplated in this regard that do not contain a significant ORF, permitting identification of the effect of gene unit length independently of

30   an effect of the expressed protein of that gene. These heterologous sequences may be inserted as an extra gene unit of various sizes, for example, from about 150 or more nts in length up to 3,000 nts or more in length. Gene unit (GU) insertions of about 1,000 or 2,000 nts in length will often substantially attenuate rHMPV candidates for the upper respiratory tract of mammalian subjects. In addition, gene unit insertions can have the dual effect of both attenuating a candidate virus and

35   inducing an immunogenic response against a second virus. Alternately, gene extensions in the 3'-noncoding region (NCR) of a HMPV gene, which cannot express additional proteins, can also be attenuating in and of themselves. Within these methods of the current disclosure, gene insertion

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length is a determinant of attenuation (see, for example, U.S. Patent Application No. 10/302,547, filed November 21, 2002 and priority U.S. Provisional Application No. 60/331,961, filed November 21, 2001; and U.S. Provisional Application No. 60/412,053, filed September 18, 2002).

Deletions, insertions, substitutions and other mutations involving changes of whole viral
5   genes or genome segments within rHMPV yield highly stable candidates, which are particularly important in the case of immunosuppressed individuals. Many of these changes will result in attenuation of resultant strains, whereas others will specify different types of desired phenotypic changes. For example, accessory (that is, not essential for *in vitro* growth) genes are excellent candidates to encode proteins that specifically interfere with host immunity (see, for example, Kato *et*
10   *al.*, EMBO. J. 16:578-87, 1997). Ablation of such genes in candidate viruses can reduce virulence and pathogenesis and/or improve immunogenicity.

In more detailed embodiments, chimeric HMPVs are constructed using a HMPV "vector" genome or antigenome that is recombinantly modified to incorporate one or more antigenic determinants of a heterologous pathogen. The vector genome or antigenome includes a partial or
15   complete HMPV genome or antigenome, which may itself incorporate nucleotide modifications such as attenuating mutations. The vector genome or antigenome is modified to form a chimeric structure through incorporation of a heterologous gene or genome segment. More specifically, chimeric HMPVs of the current disclosure are constructed through a cDNA-based virus recovery system that yields recombinant viruses that incorporate a partial or complete vector or "background" HMPV
20   genome or antigenome combined with one or more "donor" nucleotide sequences encoding the heterologous antigenic determinants. In exemplary embodiments a HMPV vector genome or antigenome is modified to incorporate one or more genes or genome segments that encode antigenic determinants of one or more heterologous negative stranded RNA viruses (for example, another HMPV, RSV, PIV, or measles virus). Thus constructed, chimeric HMPVs can elicit, for example, an
25   immune response against a specific HMPV or against a non-HMPV pathogen. Alternatively, compositions and methods are provided employing a HMPV-based chimeric virus to elicit a polyspecific immune response against multiple HPIVs, against one or more HMPVs and a non-PIV pathogen such as RSV, PIV, or measles virus.

A chimeric HMPV in this context can incorporate a partial or complete human HMPV
30   incorporating one or more heterologous polynucleotides encoding one or more antigenic determinants of the heterologous pathogen. These heterologous polynucleotides can be added to or substituted within the HMPV vector genome or antigenome to yield the chimeric HMPV recombinant. The chimeric HMPV virus thus acquires the ability to elicit an immune response in a selected host against the heterologous pathogen. In addition, the chimeric virus can exhibit other novel phenotypic
35   characteristics compared to one or both of the vector HMPV and heterologous pathogens. In addition to providing novel immunogenic characteristics, the addition or substitution of heterologous genes or genome segments within the vector HMPV strain may confer an increase or decrease in attenuation,

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growth changes, or other desired phenotypic changes as compared with the corresponding phenotypes of the unmodified vector and donor viruses.

Heterologous genes or genome segments of a different HMPV or non-HMPV virus can be added as a supernumerary genomic element to a partial or complete genome or antigenome of a

5   rHMPV. Alternatively, one or more heterologous genes or genome segments may be substituted into the rHMPV at a position corresponding to a wild type gene order position of a counterpart genes or genome segments that is deleted within the HMPV vector genome or antigenome. In yet additional embodiments, the heterologous gene or genome segment is added or substituted at a position that is more promoter-proximal or promoter-distal compared to a wild type gene order position of the

10   counterpart gene or genome segment within the vector genome or antigenome to enhance or reduce, respectively, expression of the heterologous gene or genome segment. Additional methods and compositions that are useful for engineering chimeric HMPV employ certain known techniques, for example, as disclosed for PIV viruses (Durbin *et al.*, Virology 235:323-332, 1997; Skiadopoulos *et al.*, J. Virol. 72:1762-1768, 1998; Tao *et al.*, J Virol 72:2955-2961, 1998; Skiadopoulos *et al.*, J.

15   Virol. 73:1374-1381, 1999; Skiadopoulos *et al.*, Vaccine 18:503-510, 1999; Tao *et al.*, Vaccine 17:1100-1108, 1999; Tao *et al.*, Vaccine 18:1359-1366, 2000; U.S. Patent Application Serial No. 09/083,793, filed May 22, 1998; U.S. Patent Application Serial No. 09/458,813, filed December 10, 1999; U.S. Patent Application Serial No. 09/459,062, filed December 10, 1999; U.S. Provisional Application No. 60/047,575, filed May 23, 1997 (corresponding to International Publication No. WO

20   98/53078), and U.S. Provisional Application No. 60/059,385, filed September 19, 1997).

Chimeric HMPV can also be constructed that express a chimeric protein, for example an immunogenic glycoprotein having a cytoplasmic tail and/or transmembrane domain specific to a HMPV vector fused to a heterologous ectodomain (the ectodomain being that part of a transmembrane surface protein that extends into the luminal or extracellular space) of a different

25   HMPV or non-HMPV pathogen to provide a fusion protein that elicits an immune response against the heterologous pathogen. For example, a heterologous genome segment encoding a glycoprotein ectodomain from a RSV F, SH, M2, or G glycoprotein can be joined with a genome segment encoding the corresponding HMPV glycoprotein cytoplasmic and transmembrane domains to form a chimeric glycoprotein that elicits an immune response against RSV.

30   Briefly, HMPV expressing a chimeric glycoprotein includes a major nucleocapsid protein, a nucleocapsid phosphoprotein, a large polymerase protein, and a HMPV vector genome or antigenome that is modified to encode a chimeric glycoprotein. The chimeric glycoprotein incorporates one or more heterologous antigenic domains, fragments, or epitopes of a second, antigenically distinct HMPV or non-HMPV pathogen. In one embodiment, this is achieved by substitution within the

35   HMPV vector genome or antigenome of one or more heterologous genome segments of the second virus that encodes one or more antigenic domains, fragments, or epitopes, whereby the recombinant genome or antigenome encodes the chimeric glycoprotein that is antigenically distinct from the

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parent, vector virus. In several examples, the heterologous genome segment or segments can encode a glycoprotein ectodomain or immunogenic portion or epitope thereof. The heterologous genome segment can also include other portions of the heterologous or "donor" glycoprotein, for example both an ectodomain and transmembrane region that are substituted for counterpart glycoprotein ecto-
5   and transmembrane domains in the vector genome or antigenome.

As used herein, the term "gene" generally refers to a portion of a subject genome, for example, a HMPV genome, encoding an mRNA and typically begins at the upstream end with a GS signal and ends at the downstream end with the GE signal. The term gene is also interchangeable with the term "translational open reading frame," or "ORF," particularly in the case where a protein,
10   such as the HPIV1 or HPIV3 C protein, is expressed from an additional ORF rather than from a unique mRNA. The viral genome of all mononegaviruses also contains extragenic leader and trailer regions, possessing part of the promoters required for viral replication and transcription, as well as non-coding and intergenic regions. Transcription initiates at the 3' end and proceeds by a sequential stop-start mechanism that is guided by short conserved motifs found at the gene boundaries. The
15   upstream end of each gene contains a GS signal, that directs initiation of its respective mRNA. The downstream terminus of each gene contains a GE motif that directs polyadenylation and termination. The current disclosure provides operative identification of transcription signals and insertion sites, and provides the identification of the GS, GE and potential insertion sites within the genome.

To construct chimeric HMPVs, one or more HMPV genes or genome segments can be
20   deleted, inserted or substituted in whole or in part. This means that partial or complete deletions, insertions and substitutions may include ORFs and/or cis-acting regulatory sequences of any one or more of the HMPV genes or genome segments. By "genome segment" is meant any length of continuous nucleotides from the HMPV genome, which might be part of an ORF, a gene, or an extragenic region, or a combination thereof. In several examples, at least about 10, at least about 20,
25   at least about 30, at least about 50, or at least about 100 continuous nucleotides are deleted, inserted or substituted. When a subject genome segment encodes an antigenic determinant, the genome segment encodes at least one immunogenic epitope capable of eliciting a humoral or cell mediated immune response in a mammalian host. The genome segment can also encode an immunogenic fragment or protein domain. For example, the donor genome segment can encode multiple
30   immunogenic domains or epitopes, including recombinantly synthesized sequences that comprise multiple, repeating or different, immunogenic domains or epitopes.

Chimeric HMPV can be engineered to express one or more major antigenic determinants of a wide range of non-HMPV pathogens. The methods disclosed herein are generally adaptable for incorporation of antigenic determinants from, for example, subgroup A and subgroup B RSVs,
35   HMPV, measles virus, mumps virus, human papilloma viruses, type 1 and type 2 human immunodeficiency viruses, herpes simplex viruses, cytomegalovirus, rabies virus, Epstein Barr virus, filoviruses, bunyaviruses, flaviviruses, alphaviruses and influenza viruses, among other pathogens.

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Pathogens that can be targeted for development of immunogenic compositions include viral and bacterial pathogens, as well as protozoans and multicellular pathogens. Useful antigenic determinants from many important human pathogens in this context are known or readily identified for incorporation within chimeric HMPV. Thus, major antigens have been identified for the

5   foregoing exemplary pathogens, including the measles virus HA and F proteins; the F, G, SH and M2 proteins of RSV, mumps virus HN and F proteins, human papilloma virus L1 protein, type 1 or type 2 human immunodeficiency virus gp160 protein, herpes simplex virus and cytomegalovirus gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, and gM proteins, rabies virus G protein, Epstein Barr Virus gp350 protein; filovirus G protein, bunyavirus G protein, flavivirus E and NS1 proteins, and alphavirus E

10   protein. These major antigens, as well as other antigens known in the art for the enumerated pathogens and others, are well characterized to the extent that many of their antigenic determinants, including the full length proteins and their constituent antigenic domains, fragments and epitopes, are identified, mapped and characterized for their respective immunogenic activities.

Among the numerous, exemplary mapping studies that identify and characterize major

15   antigens of diverse pathogens for use in the methods a chimeric HMPV disclosed herein are epitope mapping studies directed, for example, to immunogenic glycoproteins of PIV. Exemplifying the subject methods and tools, van Wyke Coelingh *et al.* (J. Virol. 63:375-382, 1989) described twenty-six monoclonal antibodies (MAbs) (14 neutralizing and 12 nonneutralizing) that were used to examine the antigenic structure, biological properties, and natural variation of the fusion (F)

20   glycoprotein of HPIV3. Analysis of laboratory-selected antigenic variants and of PIV3 clinical isolates indicated that the panel of MAbs recognizes at least 20 epitopes, 14 of which participate in neutralization. Competitive binding assays confirmed that the 14 neutralization epitopes are organized into three nonoverlapping principal antigenic regions (A, B, and C) and one bridge site (AB), and that the 6 nonneutralization epitopes form four sites (D, E, F, and G). Most of the

25   neutralizing MAbs were involved in nonreciprocal competitive binding reactions, suggesting that they induce conformational changes in other neutralization epitopes. These and related methods will serve to readily determine candidate antigenic determinants among heterologous viruses for expression by HMPV vectors of the current disclosure.

Other antigenic determinants for use within the current disclosure have been identified and

30   characterized for RSV. For example, Beeler *et al.*, J. Virol. 63:2941-2950, 1989, employed eighteen neutralizing monoclonal antibodies (MAbs) specific for the fusion glycoprotein of the A2 strain of RSV to construct a detailed topological and operational map of epitopes involved in RSV neutralization and fusion. Competitive binding assays identified three nonoverlapping antigenic regions (A, B, and C) and one bridge site (AB). Thirteen MAb-resistant mutants (MARMs) were

35   selected, and the neutralization patterns of the MAbs with either MARMs or RSV clinical strains identified a minimum of 16 epitopes. MARMs selected with antibodies to six of the site A and AB epitopes displayed a small-plaque phenotype, which is consistent with an alteration in a biologically

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active region of the F molecule. Analysis of MARMs also indicated that these neutralization epitopes occupy topographically distinct but conformationally interdependent regions with unique biological and immunological properties. Antigenic variation in F epitopes was then examined by using 23 clinical isolates (18 subgroup A and 5 subgroup B) in cross-neutralization assays with the 18 anti-F

5   MAbs. This analysis identified constant, variable, and hypervariable regions on the molecule and indicated that antigenic variation in the neutralization epitopes of the RSV F glycoprotein is the result of a noncumulative genetic heterogeneity. Of the 16 epitopes, 8 were conserved on all or all but 1 of 23 subgroup A or subgroup B clinical isolates. These antigenic determinants, including the full length proteins and their constituent antigenic domains, fragments and epitopes, all represent useful

10   candidates for integration within chimeric HMPV of the current disclosure to elicit novel immune responses as described above. (See also, Anderson *et al.*, J. Infect. Dis. 151:626-633, 1985; Coelingh *et al.*, J. Virol. 63:375-382, 1989; Fenner *et al.*, Scand. J. Immunol. 24:335-340, 1986; Fernie *et al.*, Proc. Soc. Exp. Biol. Med. 171:266-271, 1982; Sato *et al.*, J. Gen. Virol. 66:1397-1409, 1985; Walsh *et al.*, J. Gen. Virol. 67:505-513, 1986, and Olmsted *et al.*, J. Virol. 63:411-420, 1989).

15   To express antigenic determinants of heterologous HMPVs and non-HMPV pathogens, numerous methods and constructs are provided herein. In certain detailed embodiments, a transcription unit comprising an ORF of a gene encoding an antigenic protein (for example, an RSV F or G gene) is added to a HMPV vector genome or antigenome at various positions, yielding exemplary chimeric "vector" candidates. In exemplary embodiments, chimeric HMPVs are

20   engineered that incorporate heterologous nucleotide sequences encoding protective antigens from RSV to produce infectious, attenuated candidates (see U.S. Patent Application No. 08/720,132, filed September 27, 1996, corresponding to International Publication WO 97/12032 published April 04/03/97, and U.S. Provisional Patent Application No. 60/007,083, filed September 27, 1995; U.S. Provisional Patent Application No. 60/021,773, filed July 15, 1996; U.S. Provisional Patent

25   Application No. 60/046,141, filed May 9, 1997; U.S. Provisional Patent Application No. 60/047,634, filed May 23, 1997; U.S. Patent Application No. 08/892,403, filed July 15, 1997 corresponding to International Publication No. WO 98/02530 published on January 22, 1998; U.S. Patent Application No. 09/291,894, filed on April 13, 1999 corresponding to International Publication No. WO 00/61611 published October 19, 2000, and priority U.S. Provisional Patent Application Serial No. 60/129,006,

30   filed on April 13, 1999; U.S. Patent Application No. 09/602,212, filed June 23, 2000 and corresponding International Publication No. WO 01/04335 published on January 18, 2001, and priority U.S. Provisional Patent Application Nos. 60/129,006, filed April 13, 1999, 60/143,097, filed July 9, 1999, and 60/143,132, filed July 9, 1999; International Publication No. WO 00/61737 published on October 19, 2000; Collins *et al.*, Proc Nat. Acad. Sci. U.S.A. 92:11563-11567, 1995;

35   Bukreyev *et al.*, J. Virol. 70:6634-41, 1996, Juhasz *et al.*, J. Virol. 71:5814-5819, 1997; Durbin *et al.*, Virology 235:323-332, 1997; He *et al.* Virology 237:249-260, 1997; Baron *et al.* J. Virol. 71:1265-1271, 1997; Whitehead *et al.*, Virology 247:232-9, 1998a; Whitehead *et al.*, J. Virol. 72:4467-4471,

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1998b; Jin *et al.* Virology 251:206-214, 1998; and Whitehead *et al.*, J. Virol. 73:3438-3442, 1999, and Bukreyev *et al.*, Proc. Nat. Acad. Sci. U.S.A. 96:2367-72, 1999). Other reports and discussion incorporated or set forth herein identify and characterize RSV antigenic determinants that are useful within the current disclosure.

HMPV chimeras incorporating one or more RSV antigenic determinants, can include a HMPV vector genome or antigenome combined with a heterologous gene or genome segment encoding an antigenic RSV glycoprotein, protein domain (for example, a glycoprotein ectodomain) or one or more immunogenic epitopes. In one embodiment, one or more genes or genome segments from RSV SH, M2, F, and/or G genes is/are combined with the HMPV vector genome or antigenome to form the chimeric HMPV candidate. Certain of these constructs will express chimeric proteins, for example fusion proteins having a cytoplasmic tail and/or transmembrane domain of HMPV fused to an ectodomain of a corresponding RSV glycoprotein to yield a novel attenuated virus that optionally elicits a multivalent immune response against both HMPV and RSV.

In certain embodiments, it is useful to administer immunogenic compositions comprising a rHMPV in a predetermined schedule with one or more additional immunogenic components, for example a second immunogenic composition against RSV or PIV administered before or after the anti-HMPV composition. RSV and HPIV3 cause significant illness within the first four months of life whereas most of the illness caused by HPIV1 and HPIV2 occur after six months of age (Chanock *et al.*, in Parainfluenza Viruses, Knipe *et al.* (Eds.), pp. 1341-1379, Lippincott Williams & Wilkins, Philadelphia, 2001; Collins *et al.*, In Fields Virology, Vol. 1, pp. 1205-1243, Lippincott-Raven Publishers, Philadelphia, 1996; Reed *et al.*, J. Infect. Dis. 175:807-13, 1997). Accordingly, certain sequential immunization protocols involve administration of a immunogenic composition as described herein that elicits an immune response against HMPV before, simultaneous with (for example, as a combined immunogenic composition), or subsequent to, administration of a second immunogenic composition directed toward another virus. In one embodiment, an immunogenic composition that elicits an immune response against HMPV, or against HMPV and RSV is administered one, two or more times early in life, with the first dose administered at or before one month of age, followed by an immunogenic composition against HPIV1 and/or HPIV2 at about four and six months of age. Alternatively, it might be advantageous to administer immunogenic components against HMPV, RSV, and one or more PIVs at the same time, perhaps as a multi-component immunogenic composition.

Thus, combinatorial immunogenic compositions and coordinate immunization protocols are provided herein for multiple pathogenic agents, including HMPV, RSV, and one or more PIVs. In exemplary embodiments, these methods and formulations are temporally selected to target early immunization against HMPV, RSV and/or PIV3. One exemplary immunization sequence employs one or more live attenuated immunogenic compositions against HMPV, RSV and/or PIV3 as early as one month of age (for example, at one and two months of age) followed by mono- or bivalent PIV1

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and/or PIV2 immunogenic composition at four and six months of age. The methods disclosed herein can be used to administer multiple immunogenic compositions, including one or more chimeric HMPV candidates, coordinately, for example, simultaneously in a mixture or separately in a defined temporal sequence (for example, in a daily or weekly sequence), wherein each virus preferably

5   expresses, for example, a different heterologous protective antigen. Such a coordinate/sequential immunization strategy, which is able to induce secondary antibody responses to multiple viral respiratory pathogens, provides a highly powerful and extremely flexible immunization regimen that is driven by the need to immunize against multiple pathogens in early infancy.

As noted above, the current disclosure permits a wide range of alterations to be

10   recombinantly produced within the HMPV genome or antigenome, yielding defined mutations that specify desired phenotypic changes. Defined mutations can be introduced by a variety of conventional techniques (for example, site-directed mutagenesis) into a cDNA copy of the genome or antigenome. The use of genomic or antigenomic cDNA subfragments to assemble a complete genome or antigenome cDNA as described herein has the advantage that each region can be

15   manipulated separately, where small cDNA constructs provide for better ease of manipulation than large cDNA constructs, and then readily assembled into a complete cDNA. Thus, the complete antigenome or genome cDNA, or a selected subfragment thereof, can be used as a template for oligonucleotide-directed mutagenesis. This can be through the intermediate of a single-stranded phagemid form, such as using the MUTA-gen® kit of Bio-Rad Laboratories (Richmond, CA), or a

20   method using the double-stranded plasmid directly as a template such as the Chameleon® mutagenesis kit of Strategene (La Jolla, CA), or by the PCR employing either an oligonucleotide primer or a template which contains the mutations of interest. A mutated subfragment can then be assembled into the complete antigenome or genome cDNA. A variety of other mutagenesis techniques are known and can be routinely adapted for use in producing the mutations of interest in a

25   HMPV antigenome or genome cDNA of the current disclosure.

Thus, in one illustrative embodiment mutations are introduced by using the MUTA-gene® phagemid *in vitro* mutagenesis kit available from Bio-Rad Laboratories. In brief, cDNA encoding a HMPV genome or antigenome is cloned into the plasmid pTZ18U, and used to transform CJ236 cells (Life Technologies). Phagemid preparations are prepared as recommended by the manufacturer.

30   Oligonucleotides are designed for mutagenesis by introduction of an altered nucleotide at the desired position of the genome or antigenome. The plasmid containing the genetically altered genome or antigenome is then amplified.

Mutations can vary from single nucleotide changes to the introduction, deletion or replacement of large cDNA segments containing one or more genes or genome segments. Genome

35   segments can correspond to structural and/or functional domains, for example, cytoplasmic, transmembrane or ectodomains of proteins, active sites such as sites that mediate binding or other biochemical interactions with different proteins, epitopic sites, for example, sites that stimulate

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antibody binding and/or humoral or cell mediated immune responses, etc. Useful genome segments in this regard range from about 15-35 nucleotides in the case of genome segments encoding small functional domains of proteins, for example, epitopic sites, to about 50, about 75, about 100, about 200-500, and about 500-1,500 or more nucleotides.

5   The ability to introduce defined mutations into infectious recombinant HMPV has many applications, including the manipulation of HMPV pathogenic and immunogenic mechanisms. For example, the functions of HMPV proteins, including the N, P, M, F, M2, SH, G and/or L proteins can be manipulated by introducing mutations which ablate or reduce the level of protein expression, or which yield mutant protein. Various genome RNA structural features, such as promoters, intergenic

10   regions, and transcription signals, can also be routinely manipulated within the methods and compositions of the current disclosure. The effects of trans-acting proteins and cis-acting RNA sequences can be readily determined, for example, using a complete antigenome cDNA in parallel assays employing HMPV minigenomes (Dimock et al., J. Virol. 67:2772-8, 1993 in its entirety), whose rescue-dependent status is useful in characterizing those mutants that may be too inhibitory to

15   be recovered in replication-independent infectious virus.

Certain substitutions, insertions, deletions or rearrangements of genes or genome segments within recombinant HMPV (for example, substitutions of a genome segment encoding a selected protein or protein region, for instance a cytoplasmic tail, transmembrane domain or ectodomain, an epitopic site or region, a binding site or region, an active site or region containing an active site, etc.)

20   are made in structural or functional relation to an existing, "counterpart" gene or genome segment from the same or different HMPV or other source. Such modifications yield novel recombinants having desired phenotypic changes compared to wild type or parental HMPV or other viral strains. For example, recombinants of this type may express a chimeric protein having a cytoplasmic tail and/or transmembrane domain of one HMPV, or of a non-HMPV virus such as PIV or RSV, fused to

25   an ectodomain of a rHMPV of the current disclosure. Other exemplary recombinants of this type express duplicate protein regions, such as duplicate immunogenic regions.

As used herein, "counterpart" genes, genome segments, proteins or protein regions, are typically from heterologous sources (for example, from different HMPV genes, or representing the same (that is, homologous or allelic) gene or genome segment in different HMPV types or strains).

30   Typical counterparts selected in this context share gross structural features, for example, each counterpart can encode a comparable protein or protein structural domain, such as a cytoplasmic domain, transmembrane domain, ectodomain, binding site or region, epitopic site or region, etc. Counterpart domains and their encoding genome segments embrace an assemblage of species having a range of size and sequence variations defined by a common biological activity among the domain or

35   genome segment variants.

Counterpart genes and genome segments, as well as other polynucleotides disclosed herein for producing recombinant HMPV, often share substantial sequence identity with a selected

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polynucleotide "reference sequence," for example, with another selected counterpart sequence. As used herein, a "reference sequence" is a defined sequence used as a basis for sequence comparison, for example, a segment of a full-length cDNA or gene, or a complete cDNA or gene sequence. Generally, a reference sequence is at least 20 nucleotides in length, frequently at least 25 nucleotides

5   in length, and often at least 50 nucleotides in length. Since two polynucleotides may each (1) include a sequence (that is, a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides, and (2) can further include a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a "comparison window" to

10   identify and compare local regions of sequence similarity. A "comparison window", as used herein, refers to a conceptual segment of at least 20 contiguous nucleotide positions wherein a polynucleotide sequence may be compared to a reference sequence of at least 20 contiguous nucleotides and wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (that is, gaps) of 20 percent or less as compared to the reference sequence (which does not

15   comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith & Waterman, (Adv. Appl. Math. 2:482, 1981), by the homology alignment algorithm of Needleman & Wunsch, (J. Mol. Biol. 48:443, 1970), by the search for similarity method of Pearson & Lipman, (Proc. Natl. Acad. Sci. U.S.A. 85:2444, 1988), by computerized implementations of these

20   algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, WI), or by inspection, and the best alignment (that is, resulting in the highest percentage of sequence similarity over the comparison window) generated by the various methods is selected. The term "sequence identity" means that two polynucleotide sequences are identical (that is, on a nucleotide-by-nucleotide basis) over the window

25   of comparison. The term "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (for example, A, T, C, G, U, or I) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (that is, the window size), and multiplying the result by 100 to

30   yield the percentage of sequence identity. The terms "substantial identity" as used herein denotes a characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence that has at least 85 percent sequence identity, preferably at least 90 to 95 percent sequence identity, more usually at least 99 percent sequence identity as compared to a reference sequence over a comparison window of at least 20 nucleotide positions, frequently over a window of at least 25-50 nucleotides,

35   wherein the percentage of sequence identity is calculated by comparing the reference sequence to the polynucleotide sequence which may include deletions or additions which total 20 percent or less of

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the reference sequence over the window of comparison. The reference sequence may be a subset of a larger sequence.

In addition to these polynucleotide sequence relationships, proteins and protein regions encoded by recombinant HMPV are also typically selected to have conservative relationships, that is, to have substantial sequence identity or sequence similarity, with selected reference polypeptides. As applied to polypeptides, the term "sequence identity" means peptides share identical amino acids at corresponding positions. The term "sequence similarity" means peptides have identical or similar amino acids (that is, conservative substitutions) at corresponding positions. The term "substantial sequence identity" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 80 percent sequence identity, such as at least 90 percent sequence identity, at least 95 percent sequence identity or more (for example, 97, 98, or 99 percent sequence identity). The term "substantial similarity" means that two peptide sequences share corresponding percentages of sequence similarity. Preferably, residue positions that are not identical differ by conservative amino acid substitutions. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Exemplary conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine. Abbreviations for the twenty naturally occurring amino acids used herein follow conventional usage (Immunology - A Synthesis, 2nd ed., E.S. Golub & D.R. Gren, eds., Sinauer Associates, Sunderland, MA, 1991). Stereoisomers (for example, D-amino acids) of the twenty conventional amino acids, unnatural amino acids such as α, α-disubstituted amino acids, N-alkyl amino acids, lactic acid, and other unconventional amino acids may also be suitable components for polypeptides of the current disclosure. Examples of unconventional amino acids include: 4-hydroxyproline, γ-carboxyglutamate, ε-N,N,N-trimethyllysine, ε-N-acetyllysine, O-phosphoserine, N-acetylserine, N-formylmethionine, 3-methylhistidine, 5-hydroxylysine, ω-N-methylarginine, and other similar amino acids and imino acids (for example, 4-hydroxyproline). Moreover, amino acids may be modified by glycosylation, phosphorylation and the like.

To select candidate viruses, the criteria of viability, attenuation and immunogenicity are determined according to well-known methods. Viruses that will be most desired in immunogenic compositions must maintain viability, have a stable attenuation phenotype, exhibit replication in an immunized host (albeit at lower levels), and effectively elicit production of an immune response in a recipient sufficient to elicit a desired immune response. The recombinant HMPVs are not only viable

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and appropriately attenuated, they are more stable genetically *in vivo*--retaining the ability to

stimulate an immune response and in some instances to expand the immune response elicited by

multiple modifications, for example, induce an immune response against different viral strains or

subgroups, or to stimulate a response mediated by a different immunologic basis, for example,

5   secretory versus serum immunoglobulins, cellular immunity, and the like.

          Recombinant HMPVs can be tested in various well-known and generally accepted *in vitro*

and *in vivo* models to confirm adequate attenuation, resistance to phenotypic reversion, and

immunogenicity. In *in vitro* assays, the modified virus (for example, a multiply attenuated,

biologically derived or recombinant HMPV) is tested, for example, for temperature sensitivity of

10   virus replication, that is, ts phenotype, and for the small plaque or other desired phenotype. Modified

viruses are further tested in animal models of HMPV infection. A variety of animal models have

been described and are summarized in various references incorporated herein. HMPV model

systems, including rodents and non-human primates, for evaluating attenuation and immunogenic

activity of HMPV candidates are widely accepted in the art, and the data obtained there from

15   correlate well with HMPV infection, attenuation and immunogenicity in humans.

          In accordance with the foregoing description, the present disclosure also provides isolated,

infectious recombinant HMPV for use in immunogenic compositions. The attenuated virus which is

a component of an immunogenic composition is in an isolated and typically purified form. By

"isolated" is meant to refer to HMPV which is in other than a native environment of a wild type virus,

20   such as the nasopharynx of an infected individual. More generally, isolated is meant to include the

attenuated virus as a component of a cell culture or other artificial medium where it can be

propagated and characterized in a controlled setting. For example, attenuated HMPV can be

produced by an infected cell culture, separated from the cell culture and added to a stabilizer.

          For use in immunogenic compositions, recombinant HMPV produced according to the

25   current disclosure can be used directly in formulations, or lyophilized, as desired, using lyophilization

protocols well known to the artisan. Lyophilized virus will typically be maintained at about 4°C.

When ready for use the lyophilized virus is reconstituted in a stabilizing solution, for example, saline

or comprising SPG, Mg^{++} and HEPES, with or without adjuvant, as further described herein.

          HMPV-based immunogenic compositions contain as an active ingredient an

30   immunogenically effective amount of a recombinant HMPV produced as described herein. The

modified virus can be introduced into a host with a physiologically acceptable carrier and/or

adjuvant. Useful carriers are well known in the art, and include, for example, water, buffered water,

0.4% saline, 0.3% glycine, hyaluronic acid and the like. The resulting aqueous solutions may be

packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile

35   solution prior to administration, as mentioned above. The compositions may contain

pharmaceutically acceptable auxiliary substances as required to approximate physiological

conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and

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the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, and the like. Acceptable adjuvants include incomplete Freund's adjuvant, MPL™ (3-O-deacylated monophosphoryl lipid A; Corixa, Hamilton IN) and IL-12 (Genetics Institute, Cambridge MA), among many other suitable adjuvants well known
5　　in the art.

Upon immunization with a recombinant HMPV composition, via aerosol, droplet, oral, topical or other route, the immune system of the host responds to the immunogenic composition by producing antibodies specific for HMPV proteins, for example, F and G glycoproteins. As a result of the immunization with an immunogenically effective amount of a recombinant HMPV produced as
10　　described herein, the host becomes at least partially or completely immune to infection by the targeted HMPV or non- HMPV pathogen, or resistant to developing moderate or severe infection there from, particularly of the lower respiratory tract.

The host to which the immunogenic compositions are administered can be any mammal which is susceptible to infection by HMPV or a selected non- HMPV pathogen and which host is
15　　capable of generating an immune response to the antigens of the immunizing strain. Accordingly, methods are provided for creating immunogenic compositions for a variety of human and veterinary uses.

The compositions containing the recombinant HMPV are administered to a host susceptible to or otherwise at risk for HMPV infection to enhance the host's own immune response capabilities.
20　　Such an amount is defined to be a "immunogenically effective dose." In this use, the precise amount of recombinant HMPV to be administered within an effective dose will depend on the host's state of health and weight, the mode of administration, the nature of the formulation, etc., but will generally range from about 10^3 to about 10^7 plaque forming units (PFU) or more of virus per host, more commonly from about 10^4 to 10^6 PFU virus per host. In any event, the formulations should provide a
25　　quantity of modified HMPV of the current disclosure sufficient to elicit a detectable immune response in the host patient against the subject pathogens.

The recombinant HMPV can be combined with viruses of other HMPV serotypes or strains to elicit a desired immune response against multiple HMPV serotypes or strains. Alternatively, an immune response against multiple HMPV serotypes or strains can be achieved by combining
30　　protective epitopes of multiple serotypes or strains engineered into one virus, as described herein. Typically when different viruses are administered they will be in admixture and administered simultaneously, but they can also be administered separately. Immunization with one strain may immunize against different strains of the same or different serotype.

In some instances it may be desirable to combine the recombinant HMPV immunogenic
35　　compositions with immunogenic compositions that induce immune responses to other agents, particularly other childhood viruses. In another aspect of the current disclosure the recombinant HMPV can be employed as a vector for protective antigens of other pathogens, such as RSV, by

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incorporating the sequences encoding those protective antigens into the recombinant HMPV genome or antigenome that is used to produce infectious virus, as described herein.

In all subjects, the precise amount of recombinant HMPV administered, and the timing and repetition of administration, will be determined using conventional methods based on the patient's state of health and weight, the mode of administration, the nature of the formulation, etc. Dosages will generally range from about 10^3 to about 10^7 plaque forming units (PFU) or more of virus per patient, more commonly from about 10^4 to 10^6 PFU virus per patient. In any event, the formulations should provide a quantity of attenuated recombinant HMPV sufficient to effectively stimulate or induce an anti- HMPV or other anti-pathogenic immune response, for example, as can be determined by hemagglutination inhibition, complement fixation, plaque neutralization, and/or enzyme-linked immunosorbent assay, among other methods. In this regard, individuals are also monitored for signs and symptoms of upper respiratory illness. As with administration to chimpanzees, the attenuated virus of grows in the nasopharynx of recipients at levels approximately 10-fold or more lower than wild type virus, or approximately 10-fold or more lower when compared to levels of incompletely attenuated virus.

In neonates and infants, multiple administrations can be required to elicit sufficient levels of immunity. Administration should begin within the first month of life, and at intervals throughout childhood, such as at two months, six months, one year and two years, as necessary to maintain an immune response against native (wild type) HMPV infection. Similarly, adults who are particularly susceptible to repeated or serious HMPV infection, such as, for example, health care workers, day care workers, family members of young children, the elderly, individuals with compromised cardiopulmonary function, can require multiple immunizations to establish and/or maintain immune responses. Levels of induced immunity can be monitored by measuring amounts of neutralizing secretory and serum antibodies, and dosages adjusted or vaccinations repeated as necessary to maintain desired levels of immune response. Further, different candidate viruses may be indicated for administration to different recipient groups. For example, an engineered HMPV expressing a cytokine or an additional protein rich in T cell epitopes may be particularly advantageous for adults rather than for infants.

HMPV-based immunogenic compositions can be combined with viruses expressing antigens of another subgroup or strain of HMPV to elicit an immune response against multiple HMPV subgroups or strains. Alternatively, the candidate virus can incorporate protective epitopes of multiple HMPV strains or subgroups engineered into one HMPV clone, as described herein.

The recombinant HMPV immunogenic compositions elicit production of an immune response that reduces or alleviates serious lower respiratory tract disease, such as pneumonia and bronchiolitis when the individual is subsequently infected with wild type HMPV. While the naturally circulating virus is still capable of causing infection, particularly in the upper respiratory tract, there is a very greatly reduced possibility of rhinitis as a result of the vaccination. Boosting of resistance

by subsequent infection by wild type virus can occur. Following immunization, there are detectable levels of host engendered serum and secretory antibodies which are capable of neutralizing homologous (of the same subgroup) wild type virus *in vitro* and *in vivo*.

In one embodiment, recombinant HMPV candidates exhibit a very substantial diminution of
5   virulence when compared to wild type virus that naturally infects humans. The virus is sufficiently attenuated so that symptoms of infection will not occur in most immunized individuals. In some instances the attenuated virus can still be capable of dissemination to nonimmunized individuals. However, its virulence is sufficiently abrogated such that severe lower respiratory tract infections in the immunized host do not occur.

10   The level of attenuation of recombinant HMPV candidates can be determined by, for example, quantifying the amount of virus present in the respiratory tract of an immunized host and comparing the amount to that produced by wild type HMPV or other attenuated HMPV which have been evaluated as candidate strains. For example, the attenuated virus will have a greater degree of restriction of replication in the upper respiratory tract of a highly susceptible host, such as a
15   chimpanzee, compared to the levels of replication of wild type virus, for example, 10- to 1000-fold less. In order to further reduce the development of rhinorrhea, which is associated with the replication of virus in the upper respiratory tract, a useful candidate virus should exhibit a restricted level of replication in both the upper and lower respiratory tract. However, the attenuated viruses must be sufficiently infectious and immunogenic in humans to elicit a desired immune response in
20   immunized individuals. Methods for determining levels of HMPV in the nasopharynx of an infected host are well known in the literature and facilitated by the methods and compositions disclosed herein.

Levels of induced immunity provided by the immunogenic compositions of the current disclosure can also be monitored by measuring amounts of neutralizing secretory and serum
25   antibodies. Based on these measurements, dosages can be adjusted or immunizations repeated as necessary to maintain desired levels of immune response. Further, different candidate viruses can be advantageous for different recipient groups. For example, an engineered recombinant HMPV strain expressing an additional protein rich in T cell epitopes may be particularly advantageous for adults rather than for infants.

30   In another embodiment, the recombinant HMPV is employed as a vector for transient gene therapy of the respiratory tract. According to this embodiment the recombinant HMPV genome or antigenome incorporates a sequence that is capable of encoding a gene product of interest. The gene product of interest is under control of the same or a different promoter from that which controls HMPV expression. The infectious recombinant HMPV produced by coexpressing the recombinant
35   HMPV genome or antigenome with the N, P, L and other desired HMPV proteins, and containing a sequence encoding the gene product of interest, is administered to a patient. Administration is typically by aerosol, nebulizer, or other topical application to the respiratory tract of the patient being

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treated. Recombinant HMPV is administered in an amount sufficient to result in the expression of
therapeutic or prophylactic levels of the desired gene product. Representative gene products that may
be administered within this method are preferably suitable for transient expression, including, for
example, interleukin-2, interleukin-4, gamma-interferon, GM-CSF, G-CSF, erythropoietin, and other
5   cytokines, glucocerebrosidase, phenylalanine hydroxylase, cystic fibrosis transmembrane
conductance regulator (CFTR), hypoxanthine-guanine phosphoribosyl transferase, cytotoxins, tumor
suppressor genes, antisense RNAs, and other candidate antigens.

The following examples are provided by way of illustration, not limitation. These examples
describe the development of a novel reverse genetics system for the recovery of HMPV from cDNA,
10   and the use of this system for construction of novel recombinant HMPV candidates. Thus, the
subject matter of the current disclosure is illustrated by the following non-limiting Examples.

EXAMPLES

The mononegaviruses generally have a genome that comprises a single negative-sense
15   strand of RNA tightly encapsidated in a nucleocapsid. There is a virally encoded polymerase that
associates with the nucleocapsid and directs transcription of the genome by a process that initiates at
the 3′ end and copies the linear array of genes by a sequential stop-start mechanism that yields
subgenomic mRNAs. Replication involves the synthesis of a complete positive sense copy called the
antigenome, which in turn serves as the template for producing progeny genomes. Newly synthesized
20   viral glycoproteins are transported to and accumulate at the plasma membrane. Progeny virus is
formed when the nucleocapsid associates with these areas of modified plasma membrane and
acquires a lipid envelope by budding.

RSV is the leading cause of hospitalization for viral lower respiratory tract disease in infants
and young children, followed by HPIV3 (Collins *et al.*, 4th ed. In "Fields Virology," D. M. Knipe, P.
25   M. Howley, Eds., Vol. 1, pp. 1443-1485, Lippincott-Williams and Wilkins Publishers, Philadelphia,
2001; Crowe *et al.*, Vaccine 13:415-421, 1995; Marx *et al.*, J. Infect. Dis. 176:1423-1427, 1997).
HPIV1 and HPIV2 are the principal etiologic agents of laryngotracheobronchitis (croup), and can
also cause pneumonia and bronchiolitis (Chanock *et al.*, 4th ed. In "Fields Virology," D. M. Knipe, P.
M. Howley, Eds., Vol. 1, pp. 1341-1379, Lippincott-Williams and Wilkins Publishers, Philadelphia,
30   2001). RSV and the PIVs also are important causes of respiratory tract disease in adults. The
available evidence indicates that HMPV is also a significant agent of human respiratory tract disease,
particularly in young infants and children but occurring in all age groups (Boivin *et al.* J. Infect. Dis.
186:1330-6, 2002).

HMPV replicates slowly and inefficiently in cell culture, rendering it relatively challenging
35   to maintain and study in laboratory. In addition, trypsin reportedly must be present in the medium in
order to activate infectivity of the virus, which complicates the lengthy incubations that are necessary

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to successfully propagate the virus. Indeed, the difficulty in propagating HMPV provides an explanation for why it previously escaped detection as an important respiratory pathogen despite several decades of widespread cultivation of respiratory tract specimens in cell culture in many hospitals and research facilities across the world.

5   Methods for the production of RSV and PIV are known in the art, and compositions to induce an immune response against these viruses have been described (see Durbin *et al.*, Virology 235:323-332, 1997; Skiadopoulos *et al.*, J. Virol. 72:1762-1768, 1998; Skiadopoulos *et al.*, J. Virol. 73:1374-1381, 1999; Tao *et al.*, Vaccine 19:3620-3631, 2001; Durbin *et al.*, J. Virol. 74:6821-6831, 2000; U.S. Patent Application Serial No. 09/083,793, filed May 22, 1998; U.S. Patent Application
10   Serial No. 09/458,813, filed December 10, 1999; U.S. Patent Application Serial No. 09/459,062, filed December 10, 1999; U.S. Provisional Application No. 60/047,575, filed May 23, 1997 (corresponding to International Publication No. WO 98/53078), U.S. Provisional Application No. 60/059,385, filed September 19, 1997; U.S. Provisional Application No. 60/170,195 filed December 10, 1999; and U.S. Patent Application Serial No. 09/733,692, filed December 8, 2000 (corresponding
15   to International Publication No. WO 01/42445A2), each incorporated herein by reference). However, HMPV provides a contrasting situation in which there are no available reference reagents such as monoclonal antibodies or available reference viral strains, and no established methods for the detection and analysis of HMPV. Knowledge concerning the molecular biology of HMPV has remained rudimentary and was previously based largely on extrapolation from other viruses.
20   Because HMPV has only recently been identified and its characterization had been very incomplete, there were many challenges to generating suitably attenuated, immunogenic and genetically stable for use in immunogenic and diagnostic compositions. To facilitate these goals, it is necessary to produce recombinant infectious HMPV from cDNA to serve as a starting point for manipulations that include the development and staged introduction of predetermined attenuating mutations; the deletion,
25   modification or rearrangement of existing genes, the introduction of foreign genes, the swapping of protective antigens between strains, and the swapping of attenuating or other desired mutations between HMPV strains and between HMPV and other related viruses, and to provide additional tools to generate immunogenic compositions, vectors and immunization methods. The present disclosure describes the production of recombinant infectious HMPV from cDNA, and attenuating mutations
30   with this cDNA, as well as the specific deletions and substitutions within existing genes, the introduction of heterologous nucleic acid sequences, and additional methods and compositions that are of use in methods designed to generate an immune response against HMPV.

35   **Example 1**
               Determination of a Complete Consensus Sequence for the Genome of HMPV

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The present Example provides the complete genomic sequence of a HMPV . The subject viral strain for this analysis is strain CAN97-83 (Peret *et al.*, J. Infect. Dis. 185:1660-3, 2002), hereafter referred to as strain 83 or CAN83. The sequence was determined by direct analysis of uncloned RT-PCR products of viral RNA and thus represents a consensus or majority sequence of a
5   viable virus. The virulence, and hence wild type status, of this virus was confirmed by the ability to induce respiratory tract disease signs in chimpanzees following intranasal inoculation, as described in a following Example. The determination of a complete authentic wild type HMPV sequence is a necessary step in developing a system to produce HMPV of defined sequence and characteristics for use in immunogenic compositions and methods.
10

Cell Lines and Viruses

HEp-2 (ATCC CCL 23), Vero (ATCC CCL-81) and LLC-MK2 (ATCC CCL 7.1) cells were maintained in OptiMEM I (Invitrogen GIBCO) supplemented with 5% fetal bovine serum and, in some instances, gentamicin sulfate (50 µg/mL). BSR T7/5 cells are baby hamster kidney 21 (BHK-
15   21) cells that have been transformed and constitutively express T7 RNA polymerase (Buchholz *et al.*, J. Virol. 73:251-9). They were maintained in Glasgow MEM supplemented with glutamine and amino acids (Invitrogen) and 5% fetal bovine serum. The BSR T7/5 cells were subjected to geneticine (1 mg/ml) selection every second passage. Biological and recombinant HMPV were propagated in Vero, LLC-MK2, or BSR T7/5 cells in the absence of serum and the presence of 5
20   µg/ml of trypsin.

Virion RNA Isolation

Confluent monolayers of LLC–MK2 cells were infected with HMPV and incubated at 32°C in the presence of 5 µg/ml trypsin. 10-14 days post–infection, clarified supernatants were harvested.
25   Virion RNA (vRNA) was isolated directly from the clarified supernatants using the QIAamp viral RNA purification kit (Qiagen) according to the manufacturer's instructions. Alternatively, virus was purified by sucrose gradient centrifugation. Supernatant and infected cells were harvested by scraping with a rubber policeman, mixed with HEPES buffer and $MgSO_4$ at a final concentration of 50 mM and 0.1 M, respectively, and then submitted to three cycles of freezing and thawing. The mixture was
30   clarified by low-speed centrifugation and the virus was pelleted by centrifugation at 7000 rpm overnight at 4°C. The virus pellet was resuspended in OptiMEM (Invitrogen) containing 50 mM of HEPES buffer and 0.1 M of $MgSO_4$. The viral suspension was layered on a 30 to 60% sucrose gradient and was centrifuged for 90 min at 26,000 rpm in an SW28 rotor. The upper virus-containing fraction was collected, diluted in TEN (10 mM Tris-HCl [pH 7.4], 0.1 M NaCl, 1 mM EDTA) and
35   virus particles were pelleted by centrifugation at 25,000 rpm in an SW28 rotor for 90 min. The virus pellet was resuspended in TEN and vRNA was isolated directly from an aliquot using the RNeasy kit (Qiagen) according to the manufacturer's instructions.

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Reverse Transcription, Polymerase Chain Reaction and Nucleotide Sequencing

vRNA was reverse transcribed using specific primers generated from the published partial sequence of HMPV strain 00-1. In most cases these failed to yield RT-PCR products, presumably

5   reflecting nucleotide sequence differences between these two strains. Therefore additional primers were designed based on the regions between ORFs in the 00-1 sequence that, by analogy to other mononegaviruses, would likely contain cis-acting signals that would be more highly conserved between HMPV strains. This strategy provided RT-PCR products for strain 83 that, upon sequence analysis, provided information for designing strain 83-specific primers. Purified RNA was mixed

10   with 50 pmol of different primers in a final volume of 14 µl in water and incubated for 5 min at 70°C and 25 min at 60°C for denaturing and annealing steps. Then, 5 µl of 5X first-strand buffer II, 1 µl of 10 mM deoxynucleoside triphosphates (dNTPs), 2 µl of 0.1 M dithiothreitol, 1 µl of RNaseOUT (40 U/µl; Invitrogen) and 2 µl of Superscript II reverse transcriptase (Invitrogen) were added to the reaction. Synthesis of cDNA by RT was conducted at 44°C for 1 h and 30 min at 51°C to minimize

15   the formation of RNA secondary structures. PCR was carried out on the reverse transcribed cDNA product using 50 pmol each of specific forward and reverse primers, 5 µl of 10X Pfx amplification buffer, 1 µl of dNTP mixture, 3 µl of MgSO$_4$ and 1 µl of Platinum Pfx DNA polymerase in a total volume of 50 µl. The PCRs were conducted on a Peltier Thermal Cycler (PTC-200; MJ Research) as follows: 3 min at 94°C and then 40 s at 94°C, 40 s at 55°C and 1 min/Kb at 68°C for 30 cycles and a

20   final extension reaction for 7 min at 68°C. RT and PCR primers designed from the 00-1 strain sequence to prime within ORFs usually were not successful generating RT-PCR products from the 83 strain, presumably reflecting sequence differences relative to the 00-1 strain. Therefore, primers were designed from the semi-conserved sequence motifs flanking the viral ORFs (see Figure 8), which resulted in the successful generation of cDNA. The nucleotide sequences of cDNA products were

25   determined by direct sequence analysis of the RT–PCR products using a ABI 3100 sequencer with the Big-Dye terminator ready reaction kit v1.1 (Applied Biosystems). Finally, the sequence was assembled from overlapping RT–PCR products.

To map and sequence the 3′ end of the HMPV genome, vRNA was converted into cDNA and amplified using the 3′ RACE System for Rapid Amplification of cDNA Ends (Invitrogen, Inc.)

30   as specified by the manufacturer. Briefly, vRNA was polyadenylated at its 3′-end using poly A polymerase (Invitrogen. Inc.) followed by first–strand cDNA synthesis primed with oligo (dT) and PCR using an HMPV specific reverse primer and a forward UAP primer supplied with the kit. Then, RACE products were used as template for a nested-PCR using an internal HMPV specific reverse primer and the AUAP primer supplied with the kit to improve the specificity of the reaction. Finally,

35   the amplified cDNA nested-PCR products were sequenced directly.

To map and sequence the 5′ end of the HMPV genome, vRNA was processed by first–strand cDNA synthesis, terminal transferase tailing, and PCR amplification as specified by the 5′ RACE System for Rapid Amplification of cDNA 5′ end Version 2.0 (Invitrogen, Inc.) using the AAP primer

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supplied with the kit. Then, the amplified cDNA RACE products were used as template for a nested-PCR using an internal HMPV specific primer and the AUAP primer supplied with the kit to improve the specificity of the reaction. Finally, the amplified cDNA nested-PCR products were sequenced directly.

5   The 5′ and 3′ ends of the HMPV genome also were confirmed by genomic RNA ligation and RT-PCR followed by sequencing. Briefly, vRNA, isolated from sucrose gradient purified virus using the RNeasy kit (Qiagen), was ligated in presence of 10 U of T4 RNA ligase (Epicentre) for 3 h at 25°C and 3 h at 37°C. A cDNA corresponding to the junction of the ligated ends was amplified by RT-PCR using HMPV specific primers. The RT-PCR products were used as template for a nested-

10   PCR using HMPV internal specific primers to improve the specificity of the reaction. Finally, the nested-PCR products were cloned into a blunt vector (pSTBlue-1; Novagen) and 20 clones were sequenced, thereby confirming the length, content and sequence of the two previously-uncharacterized ends of the HMPV genome.

       The complete sequence of HMPV strain 83 was determined to be 13335 nucleotides in

15   length, and is set forth in Figures 37A-37D. This sequence was determined directly from uncloned RT-PCR products and thus represents the authentic majority, consensus sequence of a virulent, wild type virus. This discovery provides a necessary step in the development of a reverse genetics system for recovering complete infectious virus and recombinantly engineered derivatives from cloned cDNAs. A partial sequence had been published representing a portion of the genome of HMPV strain

20   00-1 (van den Hoogen et al., Virology 295:119-132, 2002).

       A genomic map for HMPV 83 is shown in FIG. 1. Compared to RSV, the prototype pneumovirus, HMPV lacks the NS1 and NS2 genes and contains a significant difference in gene order. Specifically, the SH-G gene pair precedes the F-M2 gene pair in RSV, whereas SH-G follows F-M2 in HMPV. Identification of HMPV genes was made based on the presence of ORFs whose

25   predicted protein products exhibit significant amino acid sequence relatedness with counterpart ORFs in RSV (N, P, M, F, M2-1 and L) or, in cases where sequence relatedness was unclear, gene identifications were made by genome position and by the characteristics of the predicted protein (M2-2, G, SH). In these analyses, RSV was chosen for comparison because all of its proteins have been directly identified and in most cases functions have been assigned. In a number of cases, the size of

30   the predicted HMPV protein was substantially different from that of its RSV counterpart. For example, the putative HMPV SH protein was 180% larger than that of RSV (179 amino acids compared to 64) and the HMPV G protein was 27% shorter (219 amino acids compared to 298) and lacked certain characteristic structural features such as the conserved cysteine noose (Johnson et al., Proc. Natl Acad.Sci. USA 84:5625-9, 1987) and CX3C chemokine domain (Tripp et al., Nat. Med.

35   2:732-8, 2001). Even the putative HMPV P and L proteins exhibited significant differences in length, being 22% larger and 7% shorter, respectively, than the proposed RSV counterparts.

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The ends of the HMPV genome were mapped and sequenced. The deduced sequences of the 3′ leader and 5′ trailer regions are shown in FIG. 2. This analysis ruled out the possibility that one or more additional genes might follow the L gene or precede the N gene, comparable to the NS1 and NS genes of RSV. Comparison of the leader and trailer regions of HMPV 83 with that of APV

5   (Randhawa et al., J. Virol. 71:9849-9854, 1997) and RSV (Mink et al., Virology 185:615-624, 1991) showed a high degree of sequence identity between the first 22-23 nucleotides at each end, consistent with the idea that these conserved sequences represent important functional domains. In the case of RSV, it was directly demonstrated that the first 11 nucleotides of the RSV genome represent the core promoter essential for both transcription and RNA replication (Fearns et al., J. Virol. 76:1663-1672,

10   2002).

Thus, the published partial sequence for strain 00-1 appears to lack the core element of both the genomic and antigenomic promoters, and probably lacks any HMPV promoter whatsoever. It is noteworthy that, within the first 12 nucleotides at either end of the HMPV genome, there were at least one (3′ leader) or two (5′ trailer) sequence differences compared to the other pneumoviruses. Thus,

15   the expedient of using the termini of APV or RSV to supplement the incomplete HMPV 00-1 sequence by constructing a chimeric molecule would not have yielded an authentic HMPV sequence at either end. While the functional significance of these particular differences have yet to be fully elucidated, studies with RSV showed that any point mutations within the genomic promoter affected transcription or RNA replication or both, and in most cases these effects were severe (Fearns et al., J.

20   Virol. 76:1663-1672, 2002). In any event, for the development of a genetic system to engineer recombinant HMPV for use in immunogenic compositions, it is critical to begin with a verified authentic wild type sequence, as provided herein. This sequence was used as a starting point; confirmation that the sequence indeed is authentic and encodes a functional virus is provided by the recovery of infectious, wild type-like recombinant virus. This demonstration is provided in examples

25   below.

Another important feature of mononegavirus genomic RNA is that the 3′ and 5′ ends typically exhibit terminal complementarity, meaning that the two ends are complementary when aligned in an antiparallel fashion. This reflects the presence of a conserved sequence at each end that probably reflects a conserved promoter core. This terminal complementarity is shown for HMPV 83

30   and APV in FIG. 3. For typical mononegaviruses, the two ends exhibit a high degree of complementarity for at least the first 12 nucleotides and often more, and typically within this region the complementarity is exact or has at most one mismatch. As shown in FIG. 3, the APV genome indeed has an exact match for the first 13 nucleotides whereas, surprisingly, the HMPV 83 genome has two adjacent mismatches. This further illustrates that extrapolation from other pneumoviruses to

35   develop a HMPV genetic system based on an incomplete sequence could not have yielded an authentic HMPV sequence.

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The sequence of HMPV 83 contained a number of differences compared to that published for strain 00-1. As examples, FIG. 4 shows that the SH ORF terminated four codons earlier in the 83 sequence than in that for strain 00-1, yielding a shorter SH protein. Also, the G ORF in the HMPV 83 sequence contained a deletion that shortened the G protein by 17 amino acids, and the intergenic

5   region between the F and M2 genes was 13 nucleotides for 83 instead of 41 nucleotides. The significance of differences such as shown in FIG. 4 can be directly evaluated with the reverse genetics system of this current disclosure.

The percent amino acid sequence identity between the predicted proteins of HMPV strain 83 and those of other pneumoviruses is shown in FIG. 5. It should be noted that a subsequent Example

10   provides a more extensive comparison between strain 83 and another HMPV strain, 75, that forms part of the current disclosure, demonstrating the extent of sequence diversity between and within the proposed HMPV subgroups.   It is noteworthy that, among all of the pneumoviruses shown in FIG. 5, reverse genetics systems for the recovery of complete infectious virus have been reported only in the case of bovine RSV (BRSV) and human RSV subgroup A. This is despite that fact that PVM has

15   been known for almost 65 years and has been the subject of extensive molecular analysis beginning in the late 1980's, and despite the fact that APV has been known for almost 25 years and also is the subject of molecular analysis. The fact that reverse genetics systems are not yet available despite considerable interest in their development illustrates the difficulty of producing such systems. As has already been noted, HRSV and BRSV differ from HMPV with regard to the lack of two genes (NS1

20   and NS2) and the rearrangement of other genes (SH and G). HRSV and BRSV also differ extensively from HMPV with regard to amino acid sequence identity of the predicted HMPV proteins. Three of the potential HMPV proteins (SH, G, and M2-2), share only 6-15% sequence identity with their putative HRSV counterparts, whereas low sequence identity values (for example, less than 25% identity) are generally considered at best to correspond to remote homologs or, alternatively, to

25   proteins that are unrelated.   Conversely, the highest value for percent identity is only 46%, in the case of the L protein. Thus, HMPV is quite distinct from any virus for which a reverse genetics system for the recovery of virus has been developed. It should be further noted in this context that the function of a protein in HMPV cannot be reliably predicted by extrapolation from results obtained for a related protein of a related virus, not even for a closely related virus. Briefly, there is initial

30   uncertainty in deducing proteins from nucleotide sequence. For example, despite the reported existence of an M2-2 ORF in APV, the available evidence suggests that this ORF is not expressed (Ahmadian *et al.*, J. Gen. Virol. 80:2011-2016, 1999), whereas it clearly is expressed in RSV and has been shown to have a role in regulating RNA synthesis (Bermingham and Collins, Proc. Natl. Acad. Sci. USA 96:11259-11264, 1999; Jin *et al.*, J. Virol. 74:74-82, 2000). Thus, the presence of an ORF

35   is not proof alone of significance. As another example, in the Filovirus family, both Ebola and Marburg viruses express a protein called VP30 that is highly related between the two viruses. However, these two proteins have different functions (Weik *et al.* J. Virol. 76:8532-9, 2002;

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Muhlberger *et al.*, J. Virol. 73:2333-42, 1999). These are but two examples of the uncertainty in predicting the existence and function of a protein based on nucleotide sequence alone. This highlights the importance of direct, relevant assays as provided hereon for the demonstration of protein function. These are presented in the current disclosure in two forms: (i) the development of a mini-replicon that

5   is capable of transcription and replication when supplied with appropriate viral proteins, and importantly (ii) the development of a system for the recovery of complete infectious virus completely from cDNA, which provides the basis for analysis of the function of all elements of the genome and its encoded RNA and protein products and infectious virus.

The amino acid sequence of the predicted M protein of HMPV 83 was used to estimate the

10   phylogenetic relationships between HMPV and other members of the Paramyxovirus family, as illustrated in FIG. 6. As shown in this figure, the Paramyxovirus family consists of two subfamilies Pneumovirinae and Paramyxovirinae, herein referred to as pneumoviruses and paramyxoviruses, respectively. These subfamilies are further divided into genera, the genus of HMPV being the Metapneumoviruses. A system for producing infectious recombinant virus did not exist previously

15   for any member of this genus. This analysis provided further evidence of evolutionary distance and distinction from pneumoviruses such as RSV and, in particular, from paramyxovirus such as Sendai, measles, mumps, human parainfluenza, and Newcastle disease viruses that constitute the more well-known and more extensively-characterized members of the Paramyxovirus family.

20   **Example 2**
Assembly of a Full-Length HMPV Antigenomic cDNA Clone Containing a GFP Transcription Cassette and Recovery of Infectious Recombinant HMPV

The complete, authentic, consensus sequence (FIGS. 37A-37D) determined for HMPV strain 83 provided the basis for designing a system for producing infectious recombinant virus. FIG. 7

25   illustrates the construction of a cDNA clone encoding the complete antigenome of HMPV strain 83 designed from the complete consensus sequence. Three separate cloned subgenomic fragments were created: fragment 1 contains the putative N, P and M genes and is bordered on the upstream (left) side by an added promoter for bacteriophage T7 RNA polymerase (T7pr) and on the right hand side by an *Nhe*I site that was added to the putative N-F intergenic region as a marker. The T7 promoter

30   was designed to add three nonviral G residues to the 5' end of the antigenome, a configuration chosen to improve the efficiency of the T7 promoter. Fragment 2 included the putative F, M2, SH and G genes and is bordered on the upstream side by the added *Nhe*I site and on the downstream side by a naturally-occurring *Acc*65I site. The *Nhe*I site serves as a marker to distinguish between cDNA-derived and biologically-derived HMPV. Fragment 3 consists of the putative L gene followed by half

35   of the hepatitis delta virus ribozyme (HDVribo) bordered by an *Rsr*II site that occurs naturally within the ribozyme. The sequence of each cloned cDNA fragment was confirmed. The vector for cloning and expressing the HMPV 83 antigenome cDNA is pBSKSII, which contains the hepatitis delta virus

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ribozyme followed by a terminator for T7 RNA polymerase (T7t) and was derived from vector p3/7 that was used previously for the recovery of recombinant HPIV1, HPIV2 and HPIV3 (Durbin *et al.*, Virology 235:323-332, 1997; Schmidt *et al.*, J. Virol. 74:8922-9, 2000; Skiadopoulos *et al.*, J. Virol. 77:270-9, 2003; Newman *et al.*, Virus Genes 24:77-92, 2002). This vector was modified by the

5   insertion of a polylinker containing *Ava*I, *Aat*II, *Nhe*I, and *Acc*65I sites, which in turn served to accept the fragment 1, 2 and 3 restriction sites. Specifically, the polylinker consists of the following sequence, *CCCGGG*GACGTCCTAGCTAGCTAGGGTACCCCGCTCGAG*CGGTCCG* (SEQ ID NO: 33; *Sma*I and *Rsr*II sites italicized), and was inserted between the *Sma*I and *Rsr*II sites of the vector p3/7 of Durbin *et al.* (Durbin *et al.*, Virology 235:323-332, 1997). The final cDNA encodes

10   the complete 13335-nucleotide HMPV 83 antigenome containing three added nonviral G residues at the 5′ end. The complete pHMPV-83 recombinant plasmid ("antigenome plasmid") contains 16333 bp.

It was anticipated that the poor growth and lack of established reference reagents and assays for HMPV could complicate the recovery and identification of recombinant HMPV recovered from

15   cDNA. Therefore, the strategy was adopted to modify the antigenomic cDNA to insert a foreign marker gene encoding a detectable label, whereby expression of the marker gene could be readily monitored. The gene that was chosen encodes the well-known marker or label GFP, a cnidarian protein whose presence can be visualized by green fluorescent emission when observed under a fluorescent microscope. The GFP cDNA was the Enhanced GFP cDNA of Clonetech, Inc.

20   Expression of a detectable marker in this manner, directly linked with HMPV production, makes it possible to monitor expression of GFP by recombinant HMPV in living cells without loss of viability or sterility, and to thereby detect and quantify the recovery of rHMPV at all stages of transfection and passage.

Among the obstacles to this strategy were the lack of direct information on HMPV

25   transcription and the lack of direct mapping of gene boundaries. It therefore was necessary to identify sequence that, when attached to the foreign ORF, would render it capable of being expressed during the HMPV transcriptional program. It also was necessary to identify a site within the HMPV genome that could accommodate the insertion of a foreign gene without disrupting HMPV growth. The transcription of a typical mononegavirus genome initiates at or near the 3′ end and proceeds by a

30   sequential start-stop mechanism by which the individual genes are transcribed into individual, separate mRNAs. Typically, mononegavirus transcription is guided by short sequence motifs that flank the viral genes. For example, the genes of RSV, which is the most thoroughly characterized pneumovirus, contain a highly-conserved 10-nucleotide GS signal on the upstream end of each gene and a semi-conserved 12- to 13-nucleotide GE signal at the downstream end of each gene. These

35   motifs were first noticed when RSV mRNAs were isolated from infected cells and their termini were directly mapped and sequenced. It is thought that the GS signal directs initiation of transcription of the individual gene, and the GE signal directs polyadenylation and termination. The polymerase

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remains bound to the template and crosses the intergenic region to resume transcription at the next gene. In the case of RSV, the analysis of the mRNA ends showed that the arrangement of genes was unexpectedly complex. Specifically, the GS signal of the L gene was found to differ from that of the others, and furthermore was found to be located within its upstream neighbor rather than downstream

5   of it. As another example, the M2-1 and M2-2 ORFs were found to be contained in a single mRNA rather than two. Also, there was no consistency of spacing of the RSV GS and GE signals with respect to the ORFs, such that there were instances where the GS signals overlapped the ORFs or, alternatively, were separated from the ORFs by as many as 85 nucleotides. Similarly, certain RSV GE signals overlapped the respective ORF or were separated by as many as 173 nucleotides.

10   Furthermore, the spacing between the RSV genes was irregular, ranging from the overlapped genes mentioned above to ones separated by as many as 56 nucleotides. This indicates the uncertainty of predicting gene boundaries based on the locations of putative ORFs and potential sequence motifs. However, the methods of this current disclosure provide for the direct identification of appropriate transcription signals and insertion sites.

15   The complete sequence of HMPV strain 83 was examined for potential GS and GE motifs, as shown in FIG. 8. These motifs were identified on the basis of being located between ORFs and by exhibiting partial sequence conservation among themselves. For example, the putative HMPV GS motif has the sequence GgGAcAAgTgaaaATG, where the nucleotide assignments in upper case are conserved in each of the putative genes. In sharp contrast to RSV, the putative GS signals were found

20   in close proximity to the start of their respective putative ORFs, with the putative initiation ATG codon located at positions 14-16 relative to each putative GS signal. It should be noted that the L ORF was preceded by two potential GS sequences, either of which was a reasonable fit for the proposed consensus sequence. One of these is shown in FIG. 8, while the second is located 18 nt upstream (7102-GGGCAAAACAGCATCC). Typically mononegavirus genes each have a single GS

25   site, and it is not known which one of these is functional for the HMPV L gene. However, this can now be readily determined by the methods of this current disclosure, by mapping the 5′ end of the putative L mRNA by RT-PCR, or by testing each potential GS signal for functionality in a mini-replicon system, or by destroying each potential signal in the complete antigenome and evaluating each mutant for virus recovery and efficiency of growth.

30   The putative GE motif has the sequence AGTtaattaAAAA, where the upper case assignments are conserved in each of the putative genes. The limited number of highly-conserved nucleotide assignments in these motifs made their identification speculative, particularly since not a single HMPV signal had been directly identified to use as a benchmark. While these conserved motifs appear to contain the core of the GS and GE signals, it would not be surprising to find that either

35   sequence as described here might be shortened at one or both ends by one or several nucleotides without complete loss of activity, or that one or more additional nucleotides added to either side might affect the activity of either signal. As an indication of the uncertainty of deducing transcription

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signals from sequence data alone, van den Hoogen *et al.* noted two different types of consensus sequences between genes, one of which corresponds to that noted above (van den Hoogen *et al.*, Virology 295:119-132, 2002). In this regard, these authors concluded that they could clearly identify this particular consensus sequence for two genes, F and L (although, as noted above, there would

5   appear to be two candidates to be the GS signal for the L gene), found "variants" in several other genes, failed to detect a counterpart for the G gene, and noted that "another [different] repeated sequence...was found downstream of each of the hMPV ORFs except G", raising further uncertainty as to which, if any, of these sequence motifs might function as a transcription signal. Thus, it was important to directly identify transcription signals that could render a foreign sequence competent for

10   expression by HMPV.

       In order to directly identify sequence signals that can confer gene expression, the 16-nucleotide putative GS sequence and 13-nucleotide putative GE sequence of the N gene were attached to a cDNA containing the GFP ORF. In this configuration, the ATG present in the GS signal replaced that of the GFP ORF. As shown in FIG. 9, this transcription cassette was inserted into the

15   HMPV antigenomic cDNA following nucleotide 41, resulting in an antigenome in which the GFP gene was placed first in the 5′ to 3′ order. In the corresponding HMPV-GFP genome, the GFP gene would be the most promoter-proximal of the genes. The complete sequence of the antigenomic rHMPV-GFP cDNA (FIGS. 38A-38D) and flanking plasmid regions was confirmed by sequence analysis.

20   Initially four of the ORFs of HMPV, namely the putative N, P, L and M2-1 ORFs, were selected to be expressed in separate plasmids to complement recovery. This initial recovery protocol focused on proteins predicted to be "internal" proteins (as opposed to components of the envelope). Examination of the HMPV genome identified ORFs whose potential products share 41%, 31%, 36%, and 46% identity with the RSV N, P, M2-1 and L proteins, respectively (FIG. 5), and these ORFs

25   were selected as candidates for inclusion. The putative M2-1 HMPV ORF also was included in the recovery because it is a possible internal protein, and studies with RSV, Ebola virus and Marburg virus indicate that possible M2-1 counterparts can be involved in RNA synthesis. In this regard it is noted that the presumed M2-1 counterparts appear to play different roles in Ebola and Marburg viruses despite their high degree of relatedness (Weik *et al.* J. Virol. 76:8532-9, 2002; Muhlberger *et*

30   *al.*, J. Virol. 73:2333-42, 1999). Also, the putative HMPV M2-1 protein contains a Cys-7/Cys-15/Cys-21/His-25 motif (numbered according to the complete deduced HMPV M2-1 amino acid sequence, designated herein the Cys3-His1 motif) that is a potential zinc binding domain that might indicate a role in interacting with nucleotides or nucleic acid. On the other hand, the putative M2-2 ORF was not included in the initial recovery system, and there is evidence from APV that a

35   seemingly-corresponding M2-2 ORF is not expressed (Ahmadian *et al.*, J. Gen. Virol. 80:2011-2016, 1999).

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The ORFs for the G, F and SH proteins of HMPV were not included in the initial recovery system, because these ORFs were predicted to encode transmembrane surface proteins--based on the presence of putative hydrophobic signals and anchors in the deduced amino acid sequences. Notably, the predicted HMPV F protein shares 36% amino acid sequence relatedness to that of RSV. The

5 percent amino acid identity for the putative G and SH proteins between HMPV and RSV are 15% and 6%, respectively. Despite this substantial departure in primary structure, other features of the amino acid sequences, such as the presence of potential glycosylation sites support the identification of these proteins herein. It is generally acknowledged in the field that the envelope proteins do not have a known role in genome transcription and RNA replication. This does not preclude the possibility that

10 the expression of these additional ORFs might facilitate recovery, perhaps by increasing the efficiency of virion assembly. However, an important aspect of the current disclosure involves identification of a minimal complement of support plasmids necessary for HMPV recovery. Once minimal recovery elements were shown to be successful, additional embodiments can now be constructed and evaluated to optimize the efficiency of recovery. For example, although the

15 expression of the M2-1 ORF from a separate plasmid is shown herein to be non-essential for recovery, it may nonetheless be a desired complement that could function to influence transcription or RNA replication or some other aspect of HMPV replication. Accordingly, expression plasmids were constructed in which the N, P, L and M2-1 ORFs were placed under the control of a T7 transcription promoter and terminator.

20 Support plasmids encoding putative HMPV nucleocapsid and polymerase proteins were prepared as follows (FIG. 10): to prepare a support plasmid expressing the putative N protein, vRNA was subjected to RT-PCR using a positive sense primer designed to hybridize at beginning of the putative N ORF (nucleotides 55 to 76 in the HMPV genome) and a reverse primer designed to hybridize at the end of the putative N ORF (nucleotides 1239 to 1220 in the HMPV genome). The

25 PCR product was digested with XhoI and subjected to a partial digestion with AflIII, which was done because the putative N ORF contains a naturally-occurring AflIII and a partial digest would yield some product that had been cleaved at the terminal site and not at the internal site. The products were into pTM1 (Durbin et al., Virology 235:323-332, 1997; Durbin et al., Virology 234:74-83, 1997; Elroy-Stein et al., Proc. Natl. Acad. Sci. USA. 86:6126-30, 1989) that was digested with NcoI (which

30 leaves overhangs compatible with AflIII) and XhoI, and clones containing the complete putative N ORF were selected and the sequence confirmed.

To construct a support plasmid containing the putative P ORF of HMPV (pT7-P) (FIG. 10), vRNA was subjected to RT-PCR using a positive sense primer designed to hybridize at beginning of the putative P ORF (nucleotides 1263 to 1279 in the HMPV genome) and a reverse primer designed

35 to hybridize at the end of the putative P ORF (nucleotides 2147 to 2132 in the HMPV genome). The PCR product was digested with AflIII and XhoI and cloned into pTM1 that was digested with NcoI

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and *Xho*I. In the final cloned recombinant plasmid, the sequence of the HMPV insert and flanking plasmid regions was confirmed by sequence analysis..

A comparable putative M2-1 support plasmid for HMPV (pT7-M2-1) was generated (FIG. 10). vRNA was subjected to RT-PCR using a positive sense primer designed to hybridize at

5   beginning of the putative M2-1 ORF (nucleotides 4724 to 4740 in the HMPV genome) and a reverse primer designed to hybridize at the end of the putative M2-1 ORF (nucleotides 5287 to 5272 in the HMPV genome). The PCR product was digested with *Afl*III and *Bam*HI and cloned into pTM1 that was digested with *Nco*I and *Bam*HI. In the final cloned recombinant plasmid, the sequence of the HMPV insert and flanking plasmid regions was confirmed by sequence analysis.

10   A support plasmid containing the putative L ORF of HMPV (pT7-L; FIG. 10) was made by RT-PCR amplification of two overlapping fragments and cloned separately into pTM1 vector. The first fragment was amplified by RT-PCR using a positive sense primer designed to hybridize at the beginning of the putative L ORF (nucleotides 7133 to 7157 in the HMPV genome) and a reverse primer designed to hybridize downstream to a unique *Avr*II (nucleotides 10892 to 10897 in the

15   HMPV genome) restriction site (nucleotides 11037 to 11018 in the HMPV genome), and the second using a positive sense primer designed to hybridize upstream to the unique *Avr*II site (nucleotides 10845 to 10866 in the HMPV genome) and a reverse primer designed to hybridize at the end of the putative L ORF (nucleotides 13150 to 13124 in the HMPV genome). Then, the first fragment was treated by *Bam*HI and the Klenow fragment of the *E. coli* polymerase I to generate a blunt end,

20   digested by *Avr*II and cloned into pTM1 that was treated by *Nco*I and Klenow enzyme and digested by *Avr*II. Finally, the second fragment of the putative L ORF was digested by *Avr*II and *Xho*I and cloned in the intermediate construct digested by the same enzymes, leading to the final construct pT7-L. In the final cloned recombinant plasmid, the sequence of the HMPV insert and flanking plasmid regions was confirmed by sequence analysis.

25   To provide an initial functional evaluation of these support plasmids, a mini-replicon system was developed. A cloned cDNA was constructed so that its expression from a T7 promoter yielded a negative-sense mini-replicon RNA, or minigenome, that contained, in 3′-to 5′ order: the leader end of the HMPV genome and adjoining putative N GS signal followed by a negative sense copy of the ORF encoding bacterial chloramphenicol acetyl transferase (CAT), followed by the putative L GE

30   signal and the trailer end of the HMPV genome. The correct 3′ end was generated by a HDV ribozyme as described above. In this minigenome, the CAT gene would be under the control of putative HMPV GS and GE signals, and would be expressed as a subgenomic mRNA. The minigenome plasmid was transfected into HEp-2 cells together with the N, P, M2-1 and L support plasmids with simultaneous coinfection by vTF-7, a vaccinia virus recombinant that expresses T7

35   RNA polymerase. 48 h post transfection, intracellular RNA was isolated and analyzed by Northern blot hybridization using a double stranded probe specific to the CAT cDNA. This analysis demonstrated that coexpression of the N, P and L genes with the minigenome plasmid yielded RNA

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species of the appropriate size to represent progeny minigenomes, antigenomes, and subgenomic CAT mRNA. In this assay, the further addition of the M2-1 gene did not alter the profile of RNA products. This indicated that in this assay N, P and L coexpression was sufficient to reconstitute RNA replication and transcription. These findings are in sharp contrast to RSV, where the expression of

5   M2-1 is necessary to achieve efficient synthesis of mRNA (Collins *et al.*, Proc. Natl. Acad. Sci. USA 93:81-85, 1996; Fearns and Collins, J. Virol. 73:5852-64, 1999). These preliminary results do not rule out any role for M2-1 in HMPV RNA synthesis, since it is possible that M2-1 might have one or more functions that are not required for minigenome replication and transcription but might be essential to launch infection by complete recombinant virus. The current disclosure provides the

10 methods to expeditiously evaluate the role of M2-1 (and M2-2) in viral recovery, gene expression, and replication.

To test the ability to generate infectious recombinant HMPV from cDNA, the full-length HMPV-GFP antigenome plasmid was cotransfected with the N, P, M2-1 and L plasmids into BSR T7/5 cells, which are baby hamster kidney-21 (BHK-21) cells that constitutively express T7 RNA

15 polymerase (Buchholz *et al.* J. Virol. 73:251-59, 1999). Transfections were done in 6-well dishes with 5 μg of antigenomic plasmid, 2 μg each of N and P support plasmid, and 1 μg each of M2-1 and L support plasmid per well. Transfections were done with SuperFect (Qiagen) or Lipofectamine 2000 (Invitrogen) in medium without trypsin or serum. One day post transfection trypsin was added to 5 μg/ml. Also, to maintain the cells in an active state, they typically were split and reseeded at a

20 1:3 ratio one or two days post transfection. In another, alternative permutation, fresh trypsin was added one day post transfection, the cells were incubated for one or more hours, the cells were scraped into the medium, and the total suspension was passaged to fresh LLC-MK2 or Vero cells. Approximately 12-24 h later the medium was replaced with fresh medium containing 5 μg/ml trypsin (unless otherwise noted, 5 μg/ml trypsin was the concentration that was routinely used).

25 When examined by fluorescent microscopy on successive days post-transfection, green cells were visualized that initially consisted of scattered isolated cells and subsequently formed small foci that consisted of two or more cells and exhibited cytopathic effect consistent with HMPV. When the transfection monolayer was split into new cultures, single green cells were visualized within one day and developed over successive days into multicellular syncytia. The expression of GFP was

30 monitored by fluorescent microscopy and photographed 8 days post-transfection. The nature of the cytopathology was indistinguishable from that produced by biologically-derived HMPV. The recovered virus replicated in LLC-MK2 cells with kinetics and cytopathogenicity consistent with HMPV.

The ability to monitor the infection in live cells without compromising sterility can be used

35 to expeditiously examine conditions to improve growth. For example, it was found that the replenishment of trypsin by the addition of further amounts during incubation resulted in a greater number of infected cells. In a preliminary experiment, the addition of fresh trypsin to a concentration

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of 2.5 or 5 μg/ml at two-day intervals resulted in the highest level of GFP expression and virus spread. However, the higher level also was associated with increased syncytium formation, which has the potential to cause more rapid cell death and decreased yield. Further experimentation, combined with assay of released infectious virus (an assay that also is facilitated by the expression of the GFP marker), will serve to identify the condition of incubation and level of trypsin that is optimal for the production of infectious virus. The most optimal schedule found to date has been to add 5 μg/ml fresh trypsin at intervals of 2 or 3 days.

The expression of GFP by cells infected with the recovered virus provided evidence that the virus was cDNA-derived and was not contaminating biologically-derived HMPV. Further identification of the virus as recombinant HMPV was made in two ways, by immunofluorescence and by detection of the *Nhe*I restriction site marker. In the immunofluorescence assay, antibodies from hamsters that had been infected with biologically-derived HMPV reacted specifically with cells infected with the putative recovered rHMPV-GFP. For this assay, BSR T7/5 cells were transfected for the recovery of recombinant HMPV-GFP as described above. Four days later the cells were split again at a ratio of 1:3 and incubated on a coverslip for nine more days. Incubations were in the presence of 5 μg/ml trypsin. The cells were fixed with 80% acetone for 15 min at 4°C, followed by incubation with serum from hamsters that had been infected with HMPV, followed by incubation with goat antibodies that were specific to hamster IgG and had been labeled with the fluorescent tag Alexa-488. The same field of cells was visualized by confocal microscopy with visible light and under conditions for fluorescence. This demonstrated strong fluorescence that was specific to foci in HMPV-infected cultures.

In a related assay series, RT-PCR was performed to amplify nucleotides 2719 to 3894 (numbered according to their position in the wild type HMPV sequence exclusive of GFP) in the rHMPV-GFP genomic RNA (FIG. 12A). This RT-PCR procedure yielded a product of the expected size when the template was RNA from cells infected with biologically-derived HMPV or recombinant HMPV-GFP, but only in the latter case was the cDNA cleaved by *Nhe*I, confirming the presence of the added *Nhe*I site specific to the recombinant antigenomic cDNA from which the recombinant virus was derived. Amplification of RT-PCR products was dependent on the addition of RT, indicating that the template was indeed viral RNA and not contaminating DNA. Infectious recombinant HMPV-GFP also was recovered when the M2-1 support plasmid was omitted from the panel of support plasmids. Although these results suggest that M2-1 is not required for recovery, it is possible that M2-1 is expressed from the antigenome plasmid, as was found in the case of RSV (Collins *et al.* Virology 295: 251-255, 1999). Also, it may be that co-expression of M2-1 will have some qualitative or quantitative effect on recovery. This is suggested by the observation that, in a typical experiment, three times more infectious virus was recovered in the presence of M2-1. It is believed that the minimal complement of proteins required for recovery of HMPV is N, P and L.

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The expression of the inserted GFP coding sequence also was characterized by Northern blot hybridization. Cells were infected with biologically-derived HMPV83, rHMPV, rHMPV-GFP, or were mock infected, and total intracellular RNA was isolated three days later and analyzed by Northern blot hybridization with double-stranded DNA probes specific to the GFP or M genes. As

5   shown in FIG. 12B, the GFP-specific probe hybridized only with RNA from rHMPV-GFP-infected cells (lane 4), and detected an abundant RNA band of the appropriate size to be the predicted 746-nt (exclusive of polyA) GFP mRNA transcribed from the inserted transcription cassette. This showed that the putative HMPV GS and GE transcription signals indeed functioned in the context of the foreign ORF to direct the efficient synthesis of a monocistronic GFP RNA. The GFP probe also

10   hybridized to several larger RNAs that were of low abundance and appeared to represent GFP-N, GFP-N-P and GFP-N-P-M read-through mRNAs, as well as to a large, faint band that was of the appropriate size to contain rHMPV-GFP genome and antigenome RNA. The very low levels of read-though mRNAs indicated that termination at the end of the GFP transcription cassette was very efficient, and hence an HMPV GE signal indeed had been correctly identified. Analysis with the M-

15   specific probe identified in each of the viruses bands of the appropriate sizes to be the predicted 853-nt monocistronic M mRNA as well as bands representing the P-M and N-P-M read-through mRNAs. The M probe also detected the genome and antigenome RNA band for each virus, which in the case of rHMPV-GFP would be 748 nt larger than HMPV83 or rHMPV due to the presence of the GFP transcription cassette (FIG. 12B, lanes 6, 7 and 8).

20   To test the ability to recover recombinant HMPV that lacked the GFP gene, plasmid expressing the HMPV antigenome depicted in FIG. 11 but lacking the GFP insert was transfected into BRS T7/5 cells together with N, P, M2-1 and L support plasmids. The recovery of recombinant HMPV was confirmed by the detection of viral antigen in single cells and in foci by indirect immunofluorescence assay as described above.

25   The recovered recombinant HMPV strain 83 was compared to the biologically-derived strain 83 isolate with regard to the efficiency of multi-cycle replication *in vitro*. LLC-MK2 cells were inoculated with 0.01 plaque forming units (PFU) per cell and incubated at 32°C in the presence of 5 μg/ml trypsin. Aliquots of the medium were taken at 24 h intervals and flash frozen. The aliquots were subsequently diluted in medium containing fresh trypsin and analyzed in parallel by plaque

30   assay to determine the infectivity titer. As shown in FIG. 13, the recombinant HMPV replicated efficiently over the 10 day period tested. The efficiency of replication of rHMPV was essentially indistinguishable from that of its biologically –derived counterpart. The ability to recover a wild type-like virus entirely from cloned cDNA showed that the sequences used to construct these cDNAs are fully functional, and thus define an authentic, functional, prototypic wild type HMPV and its encoded

35   proteins. This is noteworthy, since these viruses exhibit a high frequency of mutation (and thus a sequence cannot be assumed to be functional). The rHMPV antigenomic cDNA had been sequenced in its entirety following construction, as noted above, with the result that its sequence was confirmed

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to be exactly as designed. Thus, this experiment established (i) the exact genomic sequence of a prototypic HMPV, and (ii) conditions for recovering cloned cDNAs representing this sequence. This verified prototypic system provides the basis for the expedited recovery of other strains of HMPV, as well as for the systematic modification of HMPV via changes introduced into the cloned cDNA

5   intermediate. Finally, the ability to precisely reconstruct a wild type recombinant HMPV indicates the robustness and precision of the methods disclosed herein. This is further demonstrated by the ability to recovery numerous engineered derivatives described in subsequent examples.

Next, recombinant HMPV-GFP was compared to rHMPV with regard to the efficiency of multi-cycle growth *in vitro* to evaluate the effect of the addition of a transcription cassette. As shown

10   in FIG. 14, rHMPV-GFP replicated over the tested 10-day period with an efficiency that, at times, was reduced more than 10-fold compared to rHMPV. However, at the end of the 10 day period the final titers were very similar. Thus, a foreign insert can be accommodated by HMPV without a drastic effect on *in vitro* replication, such that a recombinant virus containing such an insert can be feasibly manufactured. It also is possible that such an insert will prove to be attenuated *in vivo*, as can

15   now be expeditiously determined in experiments employing experimental animals that were identified as supporting HMPV replication in experiments described in a subsequent Example.

Example 3
The Development of Recombinant HMPV Derivatives Containing Pre-determined Mutations
20   ### Specifying Desired Phenotypic Changes

The ability to produce infectious rHMPV from cDNA provides the basis for the planned introduction of mutations into infectious HMPV to develop recombinant viral candidates possessing desirable characteristics for use in immunogenic compositions, such as temperature-sensitivity, cold adaptation, host range restriction, improved replication *in vitro*, reduced reactogenicity, increased

25   safety, increased antigen expression, increased genetic or phenotypic stability, broader immunogenic coverage, increased immunogenicity, and attenuation. Mutations might be devised that have advantages in other applications as well, such as to yield increased antigen expression and/or to facilitate preparation of purified viral protein. Each antigenome can be assayed for the ability to direct the recovery of infectious virus, and recovered mutant viruses can be evaluated for *in vitro* affects on

30   growth and cytopathogenicity, temperature sensitivity, plaque morphology, and other characteristics. Appropriate mutant viruses can then be evaluated for growth efficiency, antigen expression, temperature sensitivity, plaque morphology, cytopathogenesis, attenuation, pathogenicity, tropism, genetic and phenotypic stability, immunogenicity, safety, and protective efficacy in cell culture and predictive animal models for HMPV activity in human subjects, including mice, hamsters, cotton

35   rats, and non-human primates, as well as in clinical studies.

One type of desirable mutation involves the deletion of sequence from the HMPV genome. For example, each of the HMPV genes or ORFs or parts thereof can be systematically deleted either

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in its entirety or in part, either alone or in combination with other genes or ORFs, to obtain virus with improved properties, particularly for use within immunogenic compositions. Exemplary genes or ORFs include the SH, G, M2-2 and M2-1 ORFs, but can involve any gene or ORF or genome region or part thereof, including non-ORF sequences such as noncoding genes or extragenic regions or cis-

5   acting signals in whole or in part . As an example of the strategy of deleting entire genes, antigenomic cDNAs were designed in which the putative SH and G ORFs, each with their surrounding set of putative GS and GE signals, were deleted singly or together (FIG. 15). To make these constructs, the wild type GFP was modified to insert *BsiW*I and *BsrG*I sites at positions 5459 and 6099 (numbered relative to the complete antigenome exclusive of GFP, a convention that will be

10   followed throughout this document). These positions are within the M2-SH and SH-G intergenic regions, respectively. Cleavage at either of these two sites creates the overhang GTAC, which also is the case for the naturally-occurring *Acc65*I site located at position 6959, within the G-L intergenic region (FIG. 15). Thus, the presence of these three compatible restriction sites flanking the SH and G genes facilitate deletion of either or both genes by cleavage with the appropriate pairs of enzymes

15   followed by religation. This resulted in cDNAs encoding rHMPV-GFP antigenomes that lacked the SH (ΔSH) or G (ΔG) genes or both (ΔSH/G). Each antigenomic cDNA was evaluated for the ability to direct the recovery of infectious virus in BSR-T7 cells in the presence of the N, P, L and M2-1 support plasmids. In each case, infectious virus was readily and successfully recovered. These could then be evaluated with regard to kinetics and efficiency of growth *in vitro* and in experimental

20   animals to define advantageous properties including attenuation. The presence of the expected deletion in each virus was confirmed by RT-PCR analysis of the SH-G region of each recovered genome, as shown in FIG. 16. Also, limited nucleotide sequencing of the RT-PCR products confirmed the sequences at the junctions of the deletions.

The rHMPV-GFP ΔSH, ΔG and ΔSH/G mutants were compared with rHMPV-GFP and

25   rHMPV with regard to the efficiency of multi-cycle replication LLC-MK2 cells following infection with an input of 0.01 PFU per cell (FIG. 17A). In this experiment, the gene-deletion mutants contained the GFP marker gene and thus should be compared to rHMPV-GFP. Remarkably, rHMPV-GFP containing the deletion of a single gene, either SH or G, replicated 3- to 10-fold more efficiently than rHMPV-GFP alone. Thus, these genes are not essential for replication *in vitro* and, indeed, their

30   loss improved replicative fitness *in vitro*. This may reflect a growth advantage due to the shorter length of the genome and loss of a transcriptional unit, although it also is possible that the absence of the SH or G protein might somehow improve growth. This can readily be investigated by the methods disclosed herein by ablating translation of SH and/or G by making nucleotide substitutions that remove translational start codons and introduce termination codons, thereby ablating expression of

35   the protein while maintaining the gene number, the genome length, and the number of transcribed mRNAs. rHMPV-GFP lacking both SH and G replicated with an efficiency similar to that of complete rHMPV-GFP, suggesting as one interpretation that whatever gain might be made from

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deleting genes and shortening the genome was counteracted by a slight loss of replicative fitness due to the absence of both SH and G. Thus, production of an HMPV immunogenic composition might be improved by deletion of SH or G, but deletion of both might not be optimal. Also, deletion of either gene might increase the ability of rHMPV to accommodate one or more added foreign genes. Given
5   the efficient replication of these viruses *in vitro*, it will now be possible to evaluate the effects of these gene deletions on HMPV replication, immunogenicity, and pathogenesis in experimental animals and human volunteers.

A second, parallel set of ΔSH, ΔG, and ΔSH/G mutants was made in an rHMPV strain 83 backbone lacking the GFP marker gene. Each of the viruses from this second set of mutants was
10   readily recovered and propagated *in vitro*. These could be evaluated with regard to kinetics and efficiency of growth *in vitro* and in experimental animals to define advantageous properties including immunogenicity and attenuation. While the presence of the GFP gene can facilitate characterization of mutant viruses *in vitro* and *in vivo*, the GFP gene preferably would not be included in candidates for developing immunogenic compositions. Hence, it is advantageous to be able to expeditiously
15   produce mutants with or without GFP, or both, as appropriate.

The set of rHMPV ΔSH and ΔG viruses lacking the GFP marker gene were evaluated for replication in vivo (FIG. 17B). Golden Syrian hamsters were infected intranasally with biologically-derived HMPV83, rHMPV, rHMPVΔSH, rHMPVΔG, or rHMPVΔSH/G. Six animals from each group were sacrificed three or five days later and the nasal turbinates and lungs were harvested,
20   homogenized, and analyzed by limiting dilution to determine the viral titers (FIG. 17B). There were several instances where differences in titer were observed between days 3 and 5, the most notable being the higher titers of HMPV 83, rHMPV and rHMPVΔSH in the lungs on day 3 versus day 5. However, in general the titers for the two days followed a consistent pattern. Recombinantly-derived HMPV replicated in vivo with an efficiency similar to that of its biologically-derived parent
25   HMPV83. Thus, rHMPV appeared to have wild type-like growth properties in vivo as well as in vitro and represents a suitable starting point for developing attenuated derivatives as candidate vaccines.

Interestingly, the replication of HMPV was not significantly reduced by deletion of the SH gene (FIG. 17B). Indeed, the ΔSH virus replicated marginally better than its wild type counterpart, perhaps due to the shorter genome length and reduced gene number. In contrast, deletion of the G
30   gene resulted in a 2.9 (day 5) to 3.2 (day 3) $\log_{10}$ decrease in replication in the nasal turbinates, and a 0.3 (day 5) to 2.3 (day 3) $\log_{10}$ decrease in the lungs. The rHMPVΔSH/G double-deletion virus exhibited a similar marked reduction in virus titer. Thus, these mutant viruses are highly attenuated in vivo.

In order to evaluate possible immunogenicity and protective efficacy, the rHMPV,
35   rHMPVΔSH, rHMPVΔG and rHMPVΔSH/G viruses were administered intranasally to additional hamsters (FIG. 17C). Serum samples were taken 27 days later and analyzed for the ability to neutralize HMPV infectivity in vitro (FIG. 17C). This showed that the highly attenuated ΔG and

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ΔSH/G viruses induced high titers of HMPV-neutralizing serum antibodies even though they were highly attenuated. On day 28 post immunization, the animals were challenged intranasally with wild type HMPV, and the animals were sacrificed three days later and nasal turbinates and lungs were harvested and virus titers were determined by limiting dilution (FIG. 17C). This showed that no

5   challenge virus replication could be detected in animals that had been infected with rHMPV or rHMPVΔSH. Interestingly, animals that had been infected with rHMPVΔG or rHMPVΔSH/G also did not have detectable challenge virus replication in the lungs, and only a low level of challenge virus replication was detected in the nasal turbinates. Thus, despite their strongly attenuated nature, the rHMPVΔG and rHMPVΔSH/G viruses were immunogenic and highly protective against HMPV

10 challenge. These viruses represent promising vaccine candidates. In particular, it was remarkable that deletion of the G gene would yield a promising vaccine candidate, since the G protein is presumed to be an attachment protein important in initiating infection, and the corresponding deletion in HRSV yielded a virus that did not replicate in mice (Teng et al, Virology 289, 283-296, 2001) and was over attenuated in humans (Karron et al., Proc. Natl. Acad. Sci. USA 94: 13961-13966).

15   The deletion or otherwise modification of intergenic regions of HMPV offers an important strategy for achieving improved phenotypic properties. For example, the intergenic regions of HMPV strain 83 range in length from 2 nt (N/P) to 190 nt (G/L). Previous work with RSV indicated that plaque size was decreased if a single intergenic region was longer than 100 nt in length (Bukreyev et al., J. Virol. 74:11017-26, 2000). Two of the naturally-occurring intergenic regions of

20 HMPV strain 83 are greater than 100 nt in length, namely SH/G (124 nt) and G/L (190 nt). Thus, reducing the length of these or other intergenic regions offers a strategy to improve replication *in vitro*, while increasing intergenic length offers a strategy for attenuating the virus, as desired.

As another example, recombinant HMPV was recovered in which the M2-2 ORF was silenced (FIG. 18A). The proposed M2-1 ORF initiates at nucleotide position 4724 and terminates at

25 position 5287. The proposed M2-2 ORF potentially initiates at positions 5235 or 5247 and terminates at 5450, and thus overlaps the M2-1 ORF. To create a mutant HMPV in which the M2-2 ORF is silenced, single nucleotide substitutions were introduced at positions 5236 and 5248, which removed the two potential translational start codons without changing the amino acid coding assignment in the overlapping M2-1 ORF (FIG. 18A). A third nucleotide substitution was made at position 5272, which

30 introduced a translational stop codon into the M2-2 ORF. In addition, nucleotides 5289-5440 were deleted, which deleted most of the M2-2 ORF. These changes were introduced into the wild type rHMPV antigenomic cDNA as well as into the rHMPV-GFP antigenomic cDNA. In each case, the ΔM2-2 virus was recovered successfully from cDNA. The rHMPVΔM2-2 and rHMPV-GFPΔM2-2 viruses were compared to their respective parents, rHMPV and rHMPV-GFP, with regard to multi-

35 step growth kinetics *in vitro* (FIG. 18B). This showed that each ΔM2-2 virus replicated somewhat less efficiently than its rHMPV-GFP parent at many of the time points, although by the end of the experiment at 13 hours, the titers were very similar. These mutations are of interest for vaccine

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design, since they do not greatly affect growth in vitro (which is necessary for vaccine manufacture) and are likely attenuating in vivo. They are evaluated in the hamster model noted in Fig. 17B and C, and described more completely in a subsequent Example below.Northern blot hybridization was then used to monitor the synthesis of intracellular RNA by the ΔM2-2 virus. As shown in FIG. 18C, the

5   synthesis of genome and antigenome by the ΔM2-2 virus was not greatly different than that of wild type HMPV. However, the ΔM2-2 virus directed a level of mRNA synthesis that, when normalized to the amount of genome from replicate gel lanes, was up to 8.7-fold that of wild type HMPV. The up-regulation of protein synthesis without a concomitant increase in virus replication would be highly desirable for a vaccine virus. This phenotype resembles, but is not identical to, that of the ΔM2-2

10   mutant of human RSV. For RSV, the ΔM2-2 mutant exhibited a delay in the accumulation of mRNA that was not observed here, and exhibited a delay and reduction in the accumulation of genome and antigenome that also was not observed for HMPV. Further unanticipated differences between the HMPV and RSV are described herein, namely the ability to disrupt the HMPV M2-1 ORF, or to delete M2-1 and M2-2 altogether, without loss of viral viability for HMPV whereas either mutation

15   was lethal for RSV.

       In another application, illustrated in FIG. 19, a series of single amino acid substitutions was introduced into the cysteine-histidine motif present in the predicted M2-1 protein of rHMPV-GFP. This "Cys3-His1" motif consists of three cysteine residues at M2-1 amino acid positions 7, 15, and 21, and a histidine residue at position 25 (FIG. 19). This motif is exactly conserved in RSV and other

20   pneumoviruses. In RSV, the integrity of this motif is essential for the transcriptional processivity and anti-termination function of M2-1, and is essential for the recovery of infectious recombinant virus (Hardy and Wertz J. Virol. 74:5880-5885, 2000; Tang et al., J. Virol. 75:11328-11335, 2001). The amino acid substitutions introduced into the M2-1 protein of rHMPV-GFP were as follows: Cysteine-7-Serine (C7S), Cysteine-15-Serine (C15S), and Histidine-25-Serine (H25S). As controls, two

25   additional mutants were made involving amino acids that are found in the vicinity of the motif but are not considered to be part of the motif: Tyrosine-9-Serine (Y6S) and Asparagine-25-Serine (N25S). Each of these mutants was readily recovered as infectious virus. The C7S, Y19S, C15S, N16S, H25S and ΔM2-1 mutants of rHMPV-GFP were evaluated for multi-cycle growth in LLC-MK2 cells in parallel with rHMPV and rHMPV-GFP, as shown in FIG. 20. The mutants exhibited a range of

30   growth efficiency relative to the rHMPV-GFP parent, with the Y9S mutant replicating more efficiently, the C7S mutant replicating comparably, and the others replicating somewhat less efficiently. Nonetheless, with the exception of the H25S mutant, the final titiers at 13 days were mostly comparable to that of the rHMPV-GFP parent.

       In another mutation involving the M2-1 ORF of rHMPV-GFP, the ATG translational start

35   site was changed to a TAG termination codon, as shown in FIG. 21, resulting in the mutant designated rHMPV-GFPΔM2-1. The next ATG codon is located far down the ORF, at codon 134 out of 187 codons, and hence this mutation should ablate synthesis of M2-1. In this same mutant,

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additional termination codons were introduced closely downstream in each of the three reading frames to further preclude possible ribosomal entry and passage down the ORF. The HMPV-GFP-ΔM2-1 mutant was readily recovered as infectious virus.

The ability to rapidly and effectively recover this panel of mutants in the M2-1 ORF was a further demonstration that the cDNA-based recovery system is a robust method for expeditiously developing rHMPV variants bearing predetermined changes. The finding that a number of these mutants replicated more efficiently that their direct parent rHMPV-GFP suggests that such mutants would be valuable for use in immunogenic compositions, since improved replication *in vitro* would greatly facilitate the production of rHMPV and also might be an improved source of HMPV antigen for a protein-based immunogenic composition. In these embodiments, rHMPV-GFP was used as a parent because the expression of the GFP marker facilitates *in vitro* characterization. These forms also can be directly evaluated in experimental animals, and the presence of the marker would facilitate analysis of possible effects on virus localization *in vivo*. In addition, non-GFP versions can readily be generated, as was done above for the ΔSH, ΔG and ΔSH/G viruses, which would be appropriate for clinical evaluation. The finding that the Cys3His1 motif, and the M2-1 ORF altogether, are not required for efficient growth *in vitro* was completely unanticipated, particularly since with the Cys3-His1 motif and M2-1 protein were previously shown to be essential for the recovery of infectious RSV (Tang *et al.*, J. Virol. 75:11328-11335, 2001). In addition, it was independently confirmed that mutations in the Cys3-His1 motif of RSV, or silencing the M2-1 ORF of RSV, prevented the recovery of recombinant RSV. Although the Cys3-His1 motif is not required for efficient HMPV replication in vito, it is reasonable to anticipate that this motif and this protein have been conserved in HMPV for functional reasons and do contribute to some aspect of HMPV replication or interaction with its host. Thus, it is reasonable to anticipate that some of these mutations will be found to be attenuating *in vivo* and useful for inclusion in a live attenuated HMPV immunogenic composition.

In another exemplary embodiment the HMPV M2 gene was deleted altogether from rHMPV-GFP, including its GS and GE signals and both the M2-1 and M2-2 ORFs (FIG. 22A). The resulting rHMPV-GFP-ΔM2(1+2) virus was readily recovered and propagated *in vitro*. The ΔM2-2, ΔM2-1, and ΔM2(1+2) viruses were compared with wild type HMPV with regard to the efficiency of multi-cycle replication in LLC-MK2 and Vero cells (FIG. 22B, upper and lower panels, respectively). This experiment was performed with versions of the viruses that express GFP in order to facilitate visualization of growth, but the expression of GFP was not otherwise relevant to the experiment. In LLC-MK2 cells, each of the M2 mutant viruses replicated more than 10-fold less efficiently than wild type. In contrast, in Vero cells each of the M2 deletion viruses grew to a final titer that equaled or exceeded that of wild type HMPV. One of the important differences between LLC-MK2 and Vero cells is that the latter lack the structural genes for type I interferon. Thus, this result suggested that that the M2 deletion viruses were more sensitive to interferon than wild type HMPV. In addition, the

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observation that the M2 deletion viruses replicated efficiently in Vero cells is important, since this is a cell substrate that is acceptable for preparing vaccines for humans.

To directly evaluate the interferon-sensitivity of the M2 deletion viruses, Vero cells were pre-treated overnight with a range of concentrations of type I interferon in order to induce an antiviral
5   state. The ability of the ΔM2(1+2) and ΔM2-2 viruses to replicate was evaluated in the interferon-treated cells compared to wild type HMPV and wild type HRSV. Each of the viruses used in this experiment expressed GFP for the purpose of monitoring the infections, but the presence of GFP was not otherwise relevant to the results. Cell monolayers were infected at an moi of 1 PFU per cell (or a multiplicity of infection (moi) of 0.01 in the case of RSV, reflecting its more efficient growth) and
10   incubated for 4 days. Control monolayers were mock-interferon-treated, infected and processed in parallel. The media supernatants were harvested and analyzed by plaque assay to determine virus titers. The fold reduction between each interferon-treated culture and its corresponding mock-interferon-treated control was calculated and is shown in FIG. 22C. Thus, the replication of wild type rHMPV-GFP were reduced from 5-fold to 1680-fold by increasing amounts of interferon, compared
15   to mock-interferon-treated controls. In comparison, the ΔM2(1+2) and ΔM2-2 viruses were more sensitive and were reduced 19-fold and 13-fold, respectively at the lowest concentration and were completely inhibited at the highest concentration. Viruses that exhibit increased sensitivity to interferon typically are attenuated in vivo. Thus, these results suggest that these viruses are candidates to be attenuated derivatives.

20   Another exemplary embodiment of the current disclosure involves changing gene order to achieve attenuation, increase antigen expression, or achieve some other desirable phenotype. In a subsequent Example, the F protein of HMPV is identified as a major protective antigen, and the G and SH are also identified as virion surface proteins, which makes them likely protective antigens. In the wild type gene order, SH, G and F are located at gene positions 6, 7 and 4, respectively. It is
25   anticipated that HMPV transcription will exhibit a polar gradient, in which promoter-proximal genes are expressed more efficiently than downstream ones. Thus, placement of putative protective antigen genes such as SH, G, and F proximal to the promoter, either singly, as a pair, or as a triplet, will result in more efficient expression. In this application, the genes can be moved to this position from their wild type location, such that the virus retains a single copy of each, or the genes can be inserted as a
30   second copy, such that the virus now expresses two copies of one or more of the HMPV protective antigens. The change in gene expression due to the relocation of genes or to the addition of a second copy can have other desirable effects, such as increased or decreased growth *in vitro* or *in vivo*.

Exemplifying this strategy of "shifting" gene position for developing HMPV derivatives with improved properties, the position of the SH-G gene pair was altered in rHMPV-GFP (FIG.
35   23A). In one construct, designated "Order #1", the SH-G gene pair was moved from its position following the M2 gene to a position preceding the F gene. This results in a gene order in that region of the genome that mimics that of RSV, namely: M-SH-G-F-M2-L. Hence, the positions of four

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genes were altered in "Order 1", namely SH and G (each moved upstream by two positions) and F and M2 (each moved downstream by two positions). Accordingly, any gene or combination of genes can be moved within the genome, obtaining changes in gene expression that can provide desirable phenotypes including increased antigen expression, improved growth *in vitro*, and attenuation. As shown in FIG. 23B, the kinetics and magnitude of replication of the mutant called RSV Order #1 was indistinguishable from that of its direct parent rHMPV-GFP. This showed that changes in gene order can readily be achieved, resulting in infectious virus that replicates efficiently *in vitro* and can be evaluated for desirable properties such as attenuation or improved antigen expression *in vivo*.

As another permutation shown in FIG. 23A, the SH-G pair was moved to be upstream of the F gene in rHMPV-GFP, and a second copy of the pair was inserted, resulting in the construct "Order # 2". Thus, this construct contains two copies of the SH and G genes, which are potential protective antigens. In this case, the F-M2 gene pair has been moved a total of four positions downstream relative to the viral promoter, and the L gene is now two positions further downstream compared to the parental wild type HMPV. Yet another strategy of "gene shifting" involves moving the presumed protective antigen G and F genes from their normal positions as the 7th and 4th genes, respectively, in the HMPV gene map to promoter-proximal positions. Each gene can be moved individually or as a pair, as shown in FIG. 24A. In the case of the constructs shown in FIG. 24A, the promoter-proximal insertion site will be the same as was used for insertion of the GFP transcription cassette to make the rHMPV-GFP construct (FIG. 9), from which efficient expression has been demonstrated. While these represent preferred signals and insertion sites, other signals and insertion sites can readily be evaluated and used by the methods of this current disclosure. Preferred insertion sites would be ones in proposed intergenic regions or regions of genes that are outside of the ORFs and putative transcription signals and thus more likely to accommodate engineering without affecting HMPV replication. A subsequent Example provides identification of genome regions that are poorly conserved between divergent strains of HMPV, suggesting that they are less likely to be essential, which provides further guidance to identify preferred insertion sites. The transcription cassette should preferably be designed so that, following its insertion into the antigenomic cDNA clone, the inserted ORF is flanked by a set of GS and GE signals and each gene is the backbone similarly is flanked by a set of transcription signals. In the gene-shift constructs shown in FIG. 24A, insertion of the G and/or F gene into a promoter proximal position was accompanied by deletion of the corresponding copy from the normal antigenome position: in instances where G was shifted to the first or second position, nucleotides 6101-6960 were deleted to delete G from its natural location, and in instances where F was shifted, nucleotides 3053-4710 were deleted to delete the naturally-occurring copy of F. Analysis of the multi-step growth kinetics of these viruses *in vitro* (FIG. 24B) showed that each replicated somewhat less efficiently than the wild type parent, although the Final titers of the single-gene shift mutants (rHMPV-G1 and F1) were identical to that of the rHMPV parent, while the double-gene shift mutants were only slightly lower.

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A further set of exemplary viruses were constructed in which the G and F genes were inserted into the promoter proximal positions, but the copies of the genes in their natural positions were left undisturbed, as shown in FIG. 25A. Thus, rHMPV+G1 and +F1 contains two copies of the indicated gene (note that the symbol "+" in the designation indicates that the genes were added rather

5   than "shifted"). Similarly, rHMPV+G1F2 and +F1G2 contained an additional copy each of G and F in the indicated position, and rHMPV+G1F2F3 contained an additional copy of G and two additional copies of F in the indicated positions. Interestingly, the multi-cycle growth kinetics of these viruses were somewhat reduced compared to their rHMPV parent (FIG. 25B), but among themselves the various addition mutations grew with similar kinetics. Thus, while the addition of one gene was

10   somewhat attenuating, as had also been observed with GFP, the further addition of one or two more genes had little effect, even in the case of the relatively large F gene. The level of replication in this particualr experiment had not plateaued, and the final virus yields were not determined. In these particular constructs, the added genes were of the same virus strain. However, it also should be feasible to incorporate one or more genes from a different strain, such as one possessing antigenic

15   differences. Thus, for example, a rHMPV+G1F2 virus could be made that expresses F and G pairs representing two different strains, resulting in a single bivalent recombinant virus that would elicit a broader immune response.

The presence and intactness of the additional genes in the genome of recovered rHMPV+G1F23 were investigated by RT-PCR performed on total RNA from recovered virus

20   following three passages in Vero cells. RT-PCR was performed with a forward primer hybridizing to the HMPV leader region and a reverse primer hybridizing to N sequence, which yielded a single, major band of approximately 4.1 kb, the appropriate size to contain the tandem G1F2F3 supernumerary genes (not shown). The sequence of the supernumerary G1F2F3 genes could not be determined directly by RT-PCR consensus sequencing of viral RNA because primers specific to G or

25   F would prime on each of the two copies of G and three copies of F, precluding analysis of each gene individually. Therefore, the supernumerary genes were first amplified by RT-PCR in three separate, overlapping segments. The first RT-PCR segment used a forward (positive-sense) primer (which also served as the RT primer) from the leader region and a reverse primer from the F gene (840 nt downstream of the F GS signal): although this latter primer would prime on each copy of F, priming

30   from the promoter-proximal copy (F2, numbered as in rHMPV+G1F23) would be the most efficient in combination with this forward primer, and yielded a ~1.6 kb fragment that contained the leader, the G1 gene, and about 840 bp of the F2 gene, and was purified by gel electrophoresis for sequence analysis. The second segment was amplified by RT-PCR with an RT/forward primer located about 670 nt downstream of the F GS, and a reverse primer located 554 nt downstream of the F GS:

35   although the forward primer would prime in all three copies of F and the reverse primer in all three copies of F, priming in F2 and F3 would be the only combination to result in successful amplification of a PCR fragment (~1.5 kb) that contained the downstream 978 bp part of the F2 gene, and a 554 bp

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upstream part of F3, and was purified by gel electrophoresis for sequence analysis. The third fragment was generated with an RT/forward primer from the upstream end of F (14 nt downstream the F GS signal) and a reverse primer from the upstream end of N: the forward primer would prime in all three copies of F, but priming in F3 would be the most efficient and would yield a ~1.75 kb

5   product that contained most of the F3 gene, and 120 bp of the N gene, and was purified by gel electrophoresis for sequence analysis. This provided an RT-PCR consensus sequence of the G1F2F3 supernumerary genes that was free of mutations, and all GS/GE signals and intergenic sequences were correct, indicating that these added genes were stably recovered in recombinant virus.

To determine the effect of the gene additions on viral gene expression, cells were mock-

10   infected or infected with biologically-derived HMPV83, rHMPV, or rHMPV+G1F23, and total intracellular RNA was isolated three days later and subjected to Northern blot analysis with double stranded probes to the F, G or M genes (FIG. 25C). The F probe detected a major band of the appropriate size to be the 1644-nt F mRNA, as well as a fainter band of genome and antigenome, which in the case of rHMPV+G1F23 would be 4008 nt larger compared to HMPV83 or rHMPV due

15   to the presence of the three added gene copies (FIG. 25C, lane 4). There were only trace amounts of readthrough mRNAs detected with the F probe. Phosphorimager analysis indicated that the amount of genome plus antigenome for rHPMV+G1F23 compared to HMPV83 and rHMPV was 0.9 and 0.5, respectively (corrected for the two extra copies of the F gene present in rHMPV+G1F23). In comparison, the relative amount of F mRNA for rHMPV+G1F23 compared to HMPV83 and rHMPV

20   was 6 in each case. Normalized to the respective value of genome plus antigenome, this corresponded to a 6.6- to 12-fold higher level of F mRNA for rHMPV+G1F23 compared to HMPV83 and rHMPV.

Northern blot analysis with the G probe detected, for each of the three viruses, a band of the appropriate size to be the 711-nt G mRNA as well as bands corresponding to SH-G and M-SH-G readthrough mRNAs and a band corresponding to antigenome and genome RNAs (FIG. 25C, lanes 6,

25   7 and 8). Phosphorimager analysis indicated that the amount of genome plus antigenome for rHMPV+G1F23 compared to HMPV83 and rHMPV was 1.0 and 0.5, respectively (corrected for the extra copy of the G gene present in rHMPV+G1F23), values very similar to that observed with the F probe noted above. In comparison, the relative amount of G mRNA for rHMPV-G1F23 compared to HMPV83 and rHMPV was 14-fold and 15-fold, respectively. This would correspond to a 14- to 30-

30   fold higher level of G mRNA for rHMPV+G1F23 compared to HMPV83 and rHMPV. Thus, the expression of the G and F genes by the rHMPV+G1F23 virus indeed was greatly increased.

Yet another strategy for designing improved immunogenic compositions against HMPV is to modify the ORFs encoding protective antigens so that the codon usage is consistent with efficient translation. Specifically, for many of the codons in a given ORF, the degeneracy of the genetic code

35   allows for more that one choice of codon without changing the amino acid coding assignment. In a number of cases, specific codon choices have been shown to be associated with efficient translation while, conversely, other codon choices are associated with decreased efficiency of translation. Thus,

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the ORFs encoding HMPV antigens can be re-engineered to contain codon choices consistent with efficient translation. This can be done for ORFs in their natural genome positions as well as for ORFs that have been shifted or for heterologous ORFs that have been inserted.

Another exemplary embodiment of the current disclosure involves using a single attenuated HMPV backbone to make immunogenic compositions against more than one HMPV strain, subgroup or serotype. In this application, the protective antigen genes of an attenuated derivative of HMPV, such as strain 83, are replaced singly or in toto by the corresponding genes of a heterologous HMPV. Provided that most of the attenuating mutations of the attenuated parent HMPV lie in genes other than the protective antigen genes, this results in a chimeric virus in which the attenuated backbone of the parent bears the protective antigens of the heterologous strain. This chimeric virus can then be administered on its own, or can be combined with the original parent to make a two-virus bivalent immunogenic composition against the two viruses. This process can be repeated for additional heterologous strains as necessary.

Yet another exemplary embodiment of the current disclosure involves the addition of one or more transcription cassettes encoding one or more foreign ORFs, as exemplified in Example II by GFP. In this application, the foreign gene is placed under the control of HMPV transcription signals and is inserted into the genome in a location that does not interfere with virus viability, such as an intergenic region or a gene noncoding region. In this way, a variety of foreign proteins can be expressed, such as ones encoding protective antigens of heterologous pathogens, or immunomodulatory molecules.

In yet another exemplary embodiment, rHMPV-GFP was recombinantly modified to incorporate a Phenylalanine to Leucine mutation at amino acid position 456 in the L protein, a mutation designated F456L. This particular mutation was suggested by amino acid sequence alignment as corresponding to a position that encodes attenuating mutations in other, heterologous mutant nonsegmented negative stranded RNA viruses. The sequence alignment in FIG. 26A shows a phenylalanine-521 to Leucine (F521L) substitution in the L protein specified by a mutation in the L gene of an RSV cold-passage temperature-sensitive (*cpts*) mutant *cpts*530 (Juhasz *et al.*, J. Virol. 71:5814-9, 1999). The wild type F521 residue in the RSV sequence is exactly conserved among other mononegaviruses examined and, in particular, is present in HMPV. It should be noted that the amino acid position of the corresponding residue is not the same in each L protein, reflecting differences in length as well as small deletions and insertions elsewhere in the various individual molecules. Thus, the corresponding residue is F456 in HMPV. From this comparative mapping analysis, the F456 residue in HMPV is identified as a target for either an identical, conservative, or even non-conservative amino acid substitution (for example, substitution of the F456 residue by a leucine, or by a conservative or non-conservative amino acid as compared to leucine). In this particular Example, the F456L mutation was introduced into rHMPV-GFP and was readily recovered in a recombinant virus, designated rHMPV-GFP-F456L. As shown in FIG. 27, this mutant replicated *in vitro* with an

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efficiency that was indistinguishable from that of its direct parent, rHMPV-GFP, a characteristic that is important for efficient viral recovery, evaluation and manufacture. Thus, this mutant is an excellent candidate for further evaluation to determine its phenotypic characteristics *in vitro* and *in vivo*. Further description of this general strategy, including discussion of FIGS. 26B-26F and 28, is

5   provided above, along with other strategies for the expeditious design of attenuated derivatives based on the methods of this current disclosure.

Yet another strategy for making live attenuated vaccine candidates is the "Jennerian" approach, which involves the use of a mammalian or avian virus to immunize against an antigenically related human virus. This approach is named after Edward Jenner's successful use of cowpox as the

10   initial vaccine against smallpox in humans. This approach is based on the idea that a virus that has evolved to replicate efficiently in its natural animal host often will replicate inefficiently and thus be attenuated in the non-natural human host, reflecting a host range restriction. Such a host range restriction typically is a very stable phenotype, being the aggregate effect of the many nucleotide and amino acid sequence differences between the animal and human viral counterpart. A phenotype that

15   is based on many loci would be refractory to drift.

Other examples of "Jennerian" vaccines include the use of bovine rotavirus or bovine PIV as candidate vaccines against the respective human viruses. This can involve using the animal virus as it is provided by nature, as illustrated by the use of bovine PIV directly as a vaccine (Karron *et al.*, Pediatr Infect Dis J 15:650-654, 1996). Alternatively, the virus can be modified so that some of the

20   genes are derived from the animal virus and some from the human virus. In such a case, it is preferable to have the major protective antigens be derived from the human virus, such that the immunity that is induced will be maximally effective against the human virus, and one or more other genes be derived from the animal virus such that they confer host range restriction. One such situation involves chimeras between human and bovine parainfluenza type3 viruses: in one case, the

25   attenuated bovine backbone was modified so that the protective HN and F antigen genes were replaced by those of the human virus, thus combining the attenuated bovine viral backbone with the antigenic determinants of the human virus (Schmidt *et al.*, J. Virol. 74: 8922-8929, 2000), and in a second case the human virus was attenuated by replacing a single gene, that of the nucleocapsid N protein, with that of its bovine viral counterpart (Bailly *et al.*, J. Virol. 74:3188-3195, 2000).

30   Surprisingly, each of these bovine/human chimeric viruses was satisfactorily attenuated and immunogenic in rodent and nonhuman primates, and both are being prepared for clinical trials.

FIG. 30A illustrates chimeric HMPV vaccine candidates that were constructed by individually replacing the N, P or M ORFs of HMPV with the corresponding ORFs from APV subtype C. At the amino acid level, the N, P and M genes of HMPV and APV-C are 90, 69, and 89%

35   identical, respectively. For comparison, the N protein of bovine parainfluenza type 3 virus, whose substitution into the human PIV backbone provided a promising vaccine candidate, also was within this range, being 85% identical with its human viral counterpart. Furthermore, there are A, B and D

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subtypes of APV that are less closely related to HMPV, providing a source of additional replacements ORFs whose greater degree of divergence might provide a higher degree of attenuation.

FIG. 30 Panels B, C and D illustrate the construction of chimeric N (Part B), P (Part C), and M (Part D) genes. In each panel, the upper sequence is that of a cDNA containing the native N, P or M HMPV gene, and the bottom sequence of each panel is a cDNA in which the HMPV ORF has been replaced by its APV counterpart. Both the native HMPV and chimeric HMPV/APV cDNAs have been constructed to be flanked by restriction recognition sites for enzymes (*BbsI*, *BsmBI*, *BfuAI*, as indicated) that cleave on the inner side of each recognition sequence to yield 4-nt overhangs. Since the actual cut site of these particular enzymes are not sequence specific, the cut sites can be designed to have the native HMPV sequence. This makes it possible to ligate the N, P and M genes together in a single step, as in FIG. 30E. Any combination of HMPV and HMPV/APV genes can be ligated. For example, ligation of HMPV/APV N with HMPV P and HMPV M would yield a virus in which the N ORF alone was derived from APV. Single, double, or triple ORF replacements can readily be made. This represents one strategy for introducing attenuating host range mutations, namely a strategy in which the backbone is derived from HMPV and one or more individual ORFs from APV are introduced. An alternative strategy that also can be achieved by the methods of this current disclosure involves starting with a full length APV genome and replacing the putative protective antigen genes, namely G, F and SH, by their HMPV counterparts in whatever combination is desired. This would combine the antigenic determinants of HMPV with the attenuated backbone of APV.

Example 4

Analysis of Genetic Diversity Between Distinct Antigenic Subgroups of HMPV for Development of Improved Immunogenic Compositions

When van den Hoogen *et al.* first described the isolation of HMPV, they proposed that there was significant diversity among the various isolates (also called strains) and noted that "it is tempting to speculate that these subgroups of hMPV isolates represent different serotypes of hMPV" (Nat. Med. 7:719-24, 2001). "Serotype" means that there would be insignificant antigenic cross-reactivity between HMPV subgroups following a primary immunization, and would necessitate the development of a separate vaccine for each. Even if the HMPV subgroups do exhibit antigenic cross reactivity, it could be that sufficient antigenic diversity exists such that neutralization and protection across subgroups is significantly reduced compared to within a subgroup, and it might be necessary to have both such subgroups included in an effective immunogenic composition against HMPV. Sequence diversity also was reported by Peret *et al.*, albeit following analysis of a small segment of the genome, who proposed that there may be two "major groups or lineages" of HMPV (J. Infect. Dis. 185:1660-3, 2002). This also is supported by recent sequence analysis of the N, P, M and F genes of a number of HMPV isolates (Bastien *et al.*, Virus Res. 93:51-62, 2003). The extent of sequence and antigenic diversity among circulating HMPV strains is of considerable importance to

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epidemiology, viral detection, and development of immunogenic compositions. Strains 83 and 00-1 as described herein are members of one of the proposed subgroups, and therefore a third strain was analyzed (75) that represents the second subgroup. Comparison of the three strains provided a measure of the degree of genetic diversity between subgroups (strain 83 or 00-1 versus 75) or within a subgroup (strain 83 versus 75).

A complete consensus sequence (FIGS. 39A-39D) was determined for the genome of strain 75 using the general procedures described in Example I above for strain 83. In this analysis, the sequence of the genomic termni of strain 75 were determined by 3′ and 5′ RACE as described in Example, I. Whereas strain 83 had yielded an unambiguous sequence for its entirety, strain 75 yielded a sequence in which a number of positions in the SH and G genes contained a mixture of two assignments. This suggested that the isolate was a mixture of two or more viruses that likely reflected quasispecies variants, either present in the original clinical sample or arising during *in vitro* passage. Analysis of cloned RT-PCR products spanning the SH and G genes produced two closely related sets of sequence that likely represented two different viruses. To obtain a sequence that could be unambiguously attributed to a single replication-competent HMPV 75 virus, the isolate was subjected to biological cloning by plaque isolation. Eight independent plaque-purified preparations yielded identical consensus sequences in the region of SH and G that was consistent with that of one of the two groups of cloned cDNA sequences. This sequence appears to represent the major constituent and was taken to be HMPV 75.

The genome of strain 75 is 13,280 nucleotides in length, compared to 13,335 for strain 83 and an estimated length of 13,378 for strain 00-1. The gene maps of strains 83 and 75, showing the cross-subgroup comparison, are shown in FIG. 29. Overall, the two genomes shared 80% nucleotide identity, compared to 81% between the A2 and B1 strains of RSV representing the two RSV antigenic subgroups A and B, respectively (Genbank accession numbers M74568 and AF013254, respectively). Similarly, the nucleotide sequence of isolate 75 (FIG.s 39A-39D) was 80% identical to that of isolate 00-1 (excluding the regions for which sequence was unavailable for 00-1), representing a second cross-subgroup comparison. The sequences of isolate 83 (FIG.s 37A-37D) and 00-1, representing an intra-subgroup comparison, were 92% identical. As will be detailed further herein, the level of divergence between the two HMPV subgroups bears considerable similarity to that between the two antigenic subgroups of RSV. This finding was unpredicted and is of considerable value because there is extensive information on the significance of RSV diversity for epidemiology and development of immunogenic compositions, and this provides a very useful context for consideration of the divergence between HMPV subgroups, particularly since the overall magnitude is similar. As will be noted herein, there were some differences between the diversity of HMPV versus RSV, but even here RSV provides a useful benchmark for evaluating these differences.

For most genera of the Paramyxovirus family, there is a "rule of six" whereby the nucleotide length of each genome is an even multiple of six (Kolakofsky *et al.*, J. Virol. 72:891-899, 1998). In

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many cases, the placement of cis-acting signals relative to hexamer spacing also follows a conserved pattern. However, HMPV replication and gene expression do not appear to be ruled by a phasing requirement based on the following observations: (i) the sequences for the three available isolates differ in length, as mentioned above; (ii) no integer from 2 to 9 is evenly divisible into each of the

5   three genomes, and (iii) there was no apparent pattern of phasing for the gene boundaries within or between isolates. The absence of a phasing requirement also is suggested by the ability to recover a number of recombinant viral mutants possessing a wide variety of lengths, as shown in previous Examples. The absence of such a requirement was unpredicted, and will greatly facilitate the construction of recombinant HMPVs.

10   The availability of complete genome sequences representing the two HMPV genetic subgroups provides an opportunity to examine the relatedness of the proposed HMPV transcription signals between subgroups. This is illustrated in FIG. 31, which compares the putative GS, GE and intergenic sequences between the two subgroups. The GS and GE signals exhibited extensive sequence identity among the difference genes and between the two subgroups, and a number of

15   residues were exactly conserved in all sequences. In contrast, the intergenic regions exhibited only 48% identity between subgroups, and non-coding gene regions that were not part of the putative GS and GE signal exhibited only 54% identity. This sharp distinction in the pattern of sequence identity, combined with the functional analysis for transcription activity illustrated by the the GFP expression cassette in Example II above, provides guidance and an experimental method for a clear delineation

20   of these important cis-acting sequences. Conversely, the identification of regions such as the intergenic regions and the non-translated, non-signal gene regions points out areas where the likehood is increased that modifications involved in gene insertions, gene rearrangements and other genetic engineering would be tolerated, providing guidance that will facilitate production of recombinant viruses for use in immunogenic compositions.

25   The conserved sequence at the up-stream end of each gene, which would include the GS signal, consists of 16 nucleotides, consensus: GGGACAAnTnnnAATG (FIG. 31). One unusual feature of the GS signal of all HMPV isolates sequenced to date is the presence of ATG at positions 14-16 (FIGS. 8 and 31), which initiates the major ORF. In the SH gene of strains 75 and 00-1, there is an additional upstream ATG at GS positions 8-10: however, this start site is unlikely to be used

30   because of its unfavorable sequence context. The methods of the current disclosure allow for a determination to be made whether the ATG at positions 14-16 is a functional element of the HMPV GS signal or whether the conserved spacing of this translational start site plays some other role in gene expression. This can readily be accomplished using either complete recombinant virus or the minigenome system. The downstream end of each HMPV gene is delineated by the putative 12- to

35   15-nucleotide GE motif (consensus: AGTTAnnnAAAAA), consisting of (in positive sense) a highly conserved AGTTA pentamer, followed by a poorly conserved A/T-rich trinucleotide followed by a tract of 4 to 7 A residues. A sequence that resembles the GE motif also is present near the

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downstream end of the HMPV leader region (FIG. 31), and the methods of the current disclosure will
clarify whether this functions like a GE signal.

FIG. 32 shows the percent nucleotide identity and amino acid sequence identity between the
two HMPV subgroups (strain 83 versus 75) and within a subgroup (strain 83 versus 00-1) for the
5   various individual ORFs and their encoded proteins. With regard to nucleotide sequence identity
between the two subgroups, the HMPV ORFS shared 81-87% nucleotide identity except for SH and
G, which were substantially more divergent (69% and 59% identity, respectively). This pattern
resembled that of the two subgroups of HRSV, for which the percent nucleotide identity for the
various ORFs was 81-85% identical except for the more divergent M2-2, SH and G ORFs (69%,
10   77%, and 67% identity, respectively; FIG. 32). As noted above, the intergenic regions and the
noncoding gene regions (exclusive of GS and GE signals) were 48% and 54% identical, respectively,
between subgroups, compared to values of 42% and approximately 50%, respectively, for RSV
(Johnson *et al.*, J. Gen. Virol. 69:2901-2906, 1988). Thus, the coding sequence for HMPV G was
only marginally more conserved than HMPV noncoding gene sequence in general (59% versus 54%).
15   Extensive nucleotide sequence divergence for the ORFs encoding the SH protein and, in particular,
the G protein also was observed between two strains of the same subgroup (strain 83 versus 00-1,
FIG. 32).

FIG. 32 also shows the amino acid sequence relatedness of the various HMPV proteins
between the two subgroups (strain 83 versus strain 75), and within a subgroup (strain 83 versus 00-1).
20   With regard to the total proteome, the HMPV subgroups shared 90% amino acid sequence identity,
compared to 88% identity between the RSV subgroups. With regard to individual proteins, the most
conserved HMPV proteins were N, M, F, M2-1, and L (≥94% identity between subgroups), followed
by P and M2-2 (85%-89%), followed by the divergent SH (59%) and G (37%) proteins. SH and G
are the only ones for which the percent relatedness at the amino acid level was less than at the
25   nucleotide level, suggesting that amino acid substitutions were preferentially retained.

The pattern of HMPV sequence diversity between subgroups has general similarities to that
of RSV (FIG. 32). For RSV, the "internal" virion proteins and the F protein constituted a group of
highly conserved species, while the M2-2, SH and G proteins were more divergent. Three
differences between HMPV and RSV were particularly noteworthy: (i) the HMPV M2-2 protein was
30   markedly more conserved (89% amino acid identity between subgroups) than its RSV counterpart
(61%); (ii) the HMPV F protein also was more conserved than was the case for RSV (95% identity
between subgroups versus 89%); and (iii) the HMPV SH and G proteins were markedly more
divergent than their RSV counterparts (59% and 37% identity between subgroups, respectively,
compared to 72% and 55%, respectively). FIG. 32 also compares the HMPV proteins within a
35   subgroup (strain 83 versus 00-1). For most of the proteins, the percent amino acid identity was very
high (≤95%), with the SH and G proteins being more divergent (85% and 70% identity, respectively).
The value of 70% sequence identity for G within an HMPV subgroup is much lower that the value of

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91% reported for RSV G from the analysis of seven subgroup B strains (Sullender *et al.*, J. Virol. 65:5425-5434, 1991). Thus, the HMPV SH protein and, in particular, the G protein were markedly more divergent within and between subgroups than was the case for their HRSV counterparts. This finding was unpredicted and is important because it indicates these antigens would be ones for which

5   it will be important to have both subgroups represented in an immunogenic composition once they are identified as significant protective antigens. A test for evaluating whether or not an HMPV protein is a protective antigen is presented below.

       It was also surprising to find that the HMPV M2-2 ORF and protein exhibited a high level of identity between the two putative subgroups, since M2-2 of RSV is poorly conserved (FIG. 32). In

10   the case of RSV, the M2-2 protein was identified as a functional gene product by the observation that disruption of its ORF in infectious recombinant virus resulted in altered virus growth and RNA synthesis (Bermingham and Collins, Proc. Natl. Acad. Sci. USA 96:11259-11264, 1999; Jin *et al.*, J. Virol. 74:74-78, 2000). As noted above, rHMPV in which the M2-2 ORF has rendered nonfunctional remains viable *in vitro*, although its phenotype remains to be fully defined. The high level of

15   sequence conservation for the M2-2 ORF between HMPV subgroups suggests that it encodes a significant product, the absence of which might prove to be attenuating and useful in immunogenic compositions. The RSV M2-2 ORF is expressed by a ribosomal stop-restart mechanism, whereby ribosomes that exit the M2-1 ORF apparently double back and initiate translation at the overlapping M2-2 ORF (Ahmadian *et al.*, EMBO J. 19:2681-2689). For the HMPV M2-2 ORF, there are two

20   possible methionyl translational start sites located 38 and 50 nucleotides upstream of the translational termination codon of the M2-1 ORF. These two sites are exactly conserved among all three strains compared here, and are embedded in highly conserved sequence. This is another example where the availability of the cross-subgroup comparison is useful for identifying structural features that are of potential significance, and whose possible functional roles can be tested using complete recombinant

25   virus or minigenomes by the methods of this current disclosure.

       The HMPV F, SH and G proteins appear to correspond to the three RSV surface glycoproteins F, SH and G, although there was clear sequence relatedness between HMPV and HRSV counterparts only for F (33% amino acid identity). For RSV, G and F are the only significant neutralization antigens and are the major protective antigens. The HMPV F protein is identified as a

30   neutralization and protective antigen of HMPV in an Example below. The status of the remaining HMPV proteins as potential neutralization and protective antigens can be determined by the same method. The RSV F protein has a high degree of antigenic relatedness between subgroups (Johnson *et al.*, J. Virol. 61:3163-3166), consistent with its sequence relatedness (FIG. 32), and is the major contributor to HRSV cross-subgroup neutralization and protection. For HMPV F, amino acid

35   sequence identity between subgroups was even higher than for RSV, and it is reasonable to anticipate that this similarly will make a substantial contribution to cross-neutralization and cross-protection between the HMPV subgroups. Conversely, the high degree of divergence of the G protein between

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and within HMPV subgroups likely will compromise its contribution to cross-neutralization and cross-protection to an even greater degree than is the case for RSV.

　　　　FIG. 33 shows amino acid sequence alignments between and within a subgroup for the SH (FIG. 33A) and G (FIG. 33B) proteins. The HMPV SH protein, like its RSV counterpart, is predicted

5　　to be a type II glycoprotein that is inserted in the plasma membrane by a hydrophobic signal/anchor sequence located near its amino terminus (FIG. 33A, boxed sequence), with a cytoplasmic amino terminus and an extracellular carboxy terminus. The SH proteins of strains 75, 83, and 00-1 varied in length (177, 179, and 183 amino acids, respectively; FIG. 33A) and were considerably longer than their 64- or 65-amino acid counterparts in RSV. The predicted extracellular domain has 2-4 motifs

10　　for N-linked glycosylation, one of which is conserved in all three isolates, as well as 3 or 4 potential sites for O-glycosylation (Hansen *et al.*, Glycoconj. J. 15:115-130, 1998) that are clustered within residues 75-81 in all three isolates (FIG. 33A). In addition, the SH proteins of the three isolates contain 9 or 10 cysteine residues, which are mostly in the extracellular domain, 9 of which are conserved among all three strains. The differences in length and amino acid sequence between the

15　　SH proteins of the different isolates were concentrated in the extracellular domain.

　　　　The HMPV G protein (FIG. 33B) also is a type II surface protein, bearing a general resemblance (but no significant sequence relatedness) to the G protein of RSV. The amino acid lengths of the G proteins of strains 75, 83, and 00-1 were 236, 219, and 236, considerably shorter than their 289- to 299-amino acid counterparts in RSV. The HMPV G protein, like its RSV

20　　counterpart, contains a high percentage of serine plus threonine residues (32- 35% for the three isolates, compared to a data base average of 13%) and a somewhat elevated level of proline residues (7-8.5%, compared to an average of 5%). Strains 83, -75 and 00-1 had 1, 4 and 4, respectively, potential acceptor sites for N-linked glycosylation in the ectodomain. Each HMPV G protein contained more than 40 predicted acceptor sites for O-linked carbohydrate (not shown): by analogy to

25　　RSV it is likely that not all of these are used, and usage might be heterogeneous. The serine, threonine, proline residues and the predicted sites for O-linked sugars were concentrated in the predicted extracellular domain, suggesting that this region has a long, extended, heavily glycosylated "mucin-like" structure, as is thought to be the case for RSV.

　　　　The HMPV G protein also resembles that of RSV in having most of the amino acid

30　　divergence localized to the extracellular domain (FIG. 33B). Specifically, the cytoplasmic and transmembrane domains combined were 64% and 96% identical between and within subgroups, respectively, compared to the remarkably low values for the extracellular domain of 25% and 61% identity between and within subgroups. For comparison, the value of 25% amino acid sequence identity is comparable to that observed for the attachment hemagglutinin-neuraminidase protein of

35　　PIVs from different genera, such as Genus Paramyxovirus versus Genus Rubulavirus or Avulavirus, and is substantially lower than the value of 42-66% identity observed for the influenza A hemagglutinin glycoprotein between subtypes H1, H2 and H3.

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The HMPV G protein lacks several of the prominent features of its RSV counterpart. HMPV G lacks the conserved 13-amino acid domain in the RSV G extracellular domain that is exactly conserved among all RSV strains and is partially conserved in bovine RSV (Johnson *et al.*, Proc. Natl. Acad. Sci. USA 84:5625-5629, 1987; Teng and Collins, J. Virol. 76:6164-6171, 2002). Indeed,

5   the HMPV G ectodomain did not contain more than 3 adjacent amino acids conserved among all three isolates. HMPV G also lacks the four conserved, closely-spaced cysteine residues in HRSV G that partially overlap the 13-amino acid conserved domain, form a cystine noose, and include a CX3C chemokine motif (Tripp *et al.*, Nat. Immunol. 2:732-738 2001). The three HMPV G proteins in FIG. 33B each contained only 1 or 2 cysteine residues, with the only conserved one being at the inner face

10   of the cytoplasmic domain, representing a potential acceptor site for fatty acid acylation. HMPV G also lacks a counterpart to the second methionyl translational start codon in the HRSV G ORF, which gives rise to a secreted form and is conserved between HRSV subgroups (Roberts *et al.* J. Virol. 68:4538-4546, 1994). Only isolate CAN75 has an appropriately located potential counterpart (methionine-43), and it is the fourth rather than the second methionyl residue. Thus, comparison of

15   sequence features whose presence or absence is consistent across subgroups helps guide the identification of structural features as possible targets for mutational analysis and construction of chimeric recombinant viruses for use in immunogenic compositions according to the methods of this current disclosure.

The high level of divergence in HMPV SH and G proteins is consistent with the idea that

20   there is a preference for retaining amino acid changes in these proteins. It is particularly noteworthy that the divergence was concentrated in the extracellular domain of each protein (FIG. 33A, B). A codon-by-codon examiniation of the nucleotide coding sequences for the extracellular domains of F, G and SH ORFs indicated that 75% of the codons containing a single nucleotide change were associated with amino acid substitutions, compared to 50% for SH and 7% for F. Based on the

25   findings herein, it is suggested that the extraordinary frequency of amino acid substitution per nucleotide substitution in HMPV G (and to a lesser extent SH) reflects two factors: (i) selective pressure for amino acid change, which might come from host immunity, and (ii) the ability of the protein to tolerate substitution, which might be due to its proposed extended, unfolded nature. In contrast, HMPV F appears to resemble HRSV F, which is one of the more highly conserved proteins

30   despite its status as a major HRSV neutralization and protective antigen. This likely reflects functional and structural constraints on amino acid substitution in this folded, globular glycoprotein. The time scale of nucleotide and amino acid substitution for HMPV is not known, but it was noted that, for HRSV, it is difficult to detect antigenic drift and thus the differences probably reflect a slow accumulation over years. These findings indicate that diversity in SH and G is an important

35   consideration for HMPV epidemiology, virus detection, and development of effective immunogenic compositions.

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In conclusion, the present Example defines the amount genetic diversity that exists between the two proposed HMPV genetic subgroups and shows that, overall, the diversity between subgroups is similar in magnitude to that observed between the well-characterized RSV antigenic subgroups, although certain specific differences were observed. By comparing the two divergent HMPV

5   subgroups, the identification of transcription signal motifs was further substantiated. Conversely, the identification of poorly-conserved regions such as the non-signal, non-protein-coding gene regions and the intergenic regions provides guidance in selecting these sites as being likely to be amenable to genetic manipulation. Importantly, the F glycoprotein was shown to be highly conserved between the two subgroups, which highlights the usefulness of this surface protein for incorporation in

10   recombinant HMPV and chimeric recombinant HMPV of the current disclosure for production of broadly cross-reactive immunogenic compositions. Conversely, the other two viral surface proteins, SH and G, were demonstrated to be highly divergent between the subgroups, and will therefore be employed within recombinant HMPV and chimeric recombinant HMPV to contribute to subgroup-specific, rather than cross-subgroup, immunity (although they will often be used in multivalent

15   immunogenic compositions as described herein above). The diversity of the two genetic subgroups at the antigenic level is characterized herein, and the importance of the F protein as a major neutralization and protective antigen is demonstrated.

Example 5

20   **The Use of Experimental Animals to Evaluate HMPV Infectivity and Immunogenicity**
       Generation of Hamster Polyclonal Anti-HMPV Antibodies to the 75 and 83 Strains

To demonstrate that HMPV is infectious in a rodent animal model and can elicit an immune response characterized by production of HMPV-specific antibodies in the model host, three groups of 8- to 10-week-old golden Syrian hamsters (*Mesocricetus auratus*) were infected intranasally (IN)

25   with approximately 10^3-10^4 TCID$_{50}$ of: a mixture of HMPV strains 75 and 83, HMPV 75 alone, or HMPV 83 alone. As described in the previous Example, strains 75 and 83 (also referred to as CAN75 and CAN83, respectively) represent the two genetic subgroups of HMPV and thus provide an evaluation of the biological characteristics of viruses from each subgroup and, in particular, give a measurement of the antigenic diversity between subgroups. Serum samples were collected on day 28

30   or 44 post-immunization and examined for their ability to detect HMPV infected monkey kidney cells (LLC-MK2) by indirect immunofluorescence. LLC-MK2 cells grown in OptiMEM (Invitrogen GIBCO) on glass slides were infected with a mixture of HMPV strains 75 and 83, strain 75 alone, or strain 83 alone, and were incubated at 32°C in OptiMEM medium with 5 µg/ml trypsin. At approximately 70 hours post-infection the cells were fixed and permeabilized with 3% formaldehyde

35   and 0.1% Triton-X100 in PBS, as described previously (Skiadopoulos and McBride, J. Virol. 70:1117-24, 1996). Indirect immunofluorescence was performed with pooled hamster sera from animals that had been inoculated with HMPV 75 + 83, and fluorescein (FITC)-conjugated goat anti-

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Syrian Hamster IgG antibodies (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA) were
used for indirect immunofluorescence. As a control, serum from an adult human seropositive for
HMPV was also used to detect HMPV infected cells in a comparable indirect immunofluorescence
assay. Immunofluorescence was detected and images were collected on a Leica TCS-SP2 (Leica
5   Microsystems, Mannheim, Germany). Images were processed using the Leica TCS-NT/SP software
(version 1.6.587), Imaris 3.1.1 (Bitplane AG, Zurich Switzerland), and Adobe Photoshop 5.5 (Adobe
systems). Both the human serum and the serum from hamsters infected with HMPV strains 75 and
83 were able to detect LLC-MK2 cells infected with either HMPV strain 75 or 83, demonstrating that
IN- administered HMPV is infectious and immunogenic in hamsters. Sera from animals that received
10   strain 75 alone or strain 83 alone were further analyzed as described herein.

Generation of Rabbit Polyclonal Antibodies to HMPV Strains 75 and 83

        Specific pathogen free rabbits (Harlan Sprague Dawley) were surgically implanted with
sterilized plastic wiffle balls and were immunized by injection of approximately 10^5 TCID$_{50}$ of
15   HMPV strain 75 or 83 into the wiffle ball, as described previously (Clemons *et al.*, Lab. Anim. Sci.
42:307-11, 1992). Fluid present in the chambers was collected on day 29, and the animals received
two boosts by injection of HMPV preparations that had been purified and concentrated by
centrifugation in sucrose gradient following conventional procedures: the first boost was on day 29
and the second was on day 57. Fluid present in the chambers was collected again on day 71. These
20   sera were analyzed as described herein.

Virus Titration and Serum Antibody Infectivity Neutralization Assays

        Viral titer was quantified by serial 10-fold dilutions of virus applied to LLC-MK2
monolayer cultures on 96-well plates that were then incubated for 7 days at 37°C in OptiMEM
25   medium with 5 µg/ml added trypsin, as described previously (Newman *et al.*, Virus Genes 24:77-92,
2002). Virus infected monolayers were detected by incubation with hamster polyclonal antiserum,
which had been raised by IN infection with a mixture of strains 75 and 83 as described above,
followed by a second antibody consisting of peroxidase-conjugated rabbit anti-Syrian hamster IgG.
Bound antibody-antigen complexes were detected by immunoperoxidase staining achieved with ECL
30   chromogenic substrate.

        The susceptibility of HMPV to neutralization by HMPV-specific antiserum was determined
using an endpoint dilution neutralization assay performed with heat inactivated hamster, rabbit or
chimpanzee sera. The hamster and rabbit sera were prepared as described above. The chimpanzee
sera were from 31 caged animals, as described further herein. Briefly, replicate aliquots each
35   containing approximately 100-200 TCID$_{50}$ of HMPV 75 or 83 were mixed with aliquots from a
dilution series prepared from a heat-inactivated serum sample and incubated at 37°C for 1 hr. The
virus-antiserum mixtures were then transferred to LLC-MK2 monolayers in 96-well plates and the

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cells were incubated for 1 hour at 32°C. The monolayers were washed two times with OptiMEM to remove serum and were overlaid with OptiMEM supplemented with 5µg/ml added trypsin. The cells were then incubated for 7 days at 32°C and infected monolayers were detected by immunoperoxidase staining with the polyclonal hamster anti-HMPV 75+83 antiserum and peroxidase-conjugated rabbit

5   anti-Syrian hamster IgG and ECL chromogenic substrate, as described above. The neutralization titer is read as the highest dilution of antibody at which the cultures were 50% reduced for infection. This assay was used to determine the HMPV neutralization titer of serum from infected animals, and also was used to screen animals to determine whether they had previously been exposed to HMPV.

With the availability of the HMPV-GFP virus, the neutralization assay can now be

10   simplified. In this case, replicate aliquots of HMPV-GFP virus (approximately 100-200 TCID$_{50}$)· would be mixed with aliquots of a dilution series of serum as above, incubated for 1 hour at 32°C, and transferred to LLC-MK2 cells. The cells would be washed to remove serum, overlaid with OptiMEM supplemented with 5µg/ml added trypsin, and incubated for 7 days at 32°C. Infected cells could be visualized directly by fluorescent microscopy. The neutralization endpoint would be .   .

15   indicated by the appearance of green fluorescence, indicative of infection. Many of the details of this assay can be varied as necessary, such as the period of incubation of HMPV-GFP and antibody, the choice of cell substrate, and the period of incubation of the cells. In particular, the use of HMP-GFP means that the cells can be monitored by fluorescent microscopy to select an optimal time at which to read the assay. The efficiency of virus titration was compared by visualization of GFP verus

20   immunostaining and found to be identical. However, detection by GFP was easier because the progression of the virus infection can be monitored and the plates read at an optimal time. In contrast, immunostaining can only be done once, and the timing is determined by estimating cytopathology by eye, which is more qualitative and not as precise. A further advantage is that GFP can be read directly, whereas immunostaining is a multi-step, time-consuming process requiring antibodies and

25   development reagents. Furthermore, the washing steps need in immunostaining frequently result in damage to or removal of the cell monolayer, which can complicate or ruin the assay. Thus, using rHMPV-GFP as an indicator virus for antibody titration (or evaluation of antiviral agents) provides a better method for titration.

30   *Evaluation of the Antigenic Relatedness of Strains 83 and 75*

As described in the previous Example, it has been suggested that HMPV isolates can be segregated into two genetic subgroups (van den Hoogen *et al.*, Nat. Med. 7:719-24; Peret *et al.*, J. Infect. Dis. 185:1660-3, 2002). The previous Example described in detail the level of genetic diversity between the two proposed subgroups. The present Example (and also Example VI)

35   describes the amount of antigenic difference between the two subgroups. The extent of antigenic diversity among circulating HMPV strains is of considerable importance to epidemiology and for development of effective immunogenic compositions.

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The antigenic relatedness between strains 75 and 83 was investigated by a cross neutralization study. As described above, antiserum was raised in rabbits by parenteral immunization of gradient-purified HMPV 75 or 83 followed by a boost of the same material, with serum obtained following each step. In addition, as described above, hamsters were infected IN with strain 75 or 83 and sera were obtained. These individual sera were assayed for the ability to neutralize both strain 75 and 83. Thus, this would compare the ability of antibodies from an animal exposed to either strain to neutralize the infectivity of the homologous strain versus its ability to neutralize the heterologous strain. If extensive antigenic differences exit, each serum should be much less effective in neutralizing the heterologous strain compared to the homologous strain.

As shown in Table 1, hamsters were immunized by intranasal infection with strain 83 or 75 and the sera were collected on day 44 and evaluated for the ability to neutralize each strain *in vitro*. Of the eleven animals that were infected with strain 75, there was no consistent pattern in which the homologous 75 strain was neutralized more efficiently than the heterologous 83 strain: four animals neutralized the homologous strain more efficiently (up to 3.0 log_2 difference, with animal 2164 exhibiting the greatest difference), but one (animal 2168) showed no difference and the remainder neutralized the homologous strain less efficiently (up to 3.0 log_2 difference, animal 2174). Conversely, all ten animals infected with strain 83 yielded sera that neutralized the homologous 83 strain more efficiently than the heterologous 75 strain, with a difference ranging from 0.4 log_2 (animal 2203) to 3.4 log_2 (animal 2201). Thus, each of the HMPV strains representing the two heterologous subgroups induced antibodies that efficiently neutralized the homologous and heterologous viruses, although antibodies from animals infected with strain 83 appeared to be somewhat less effective in neutralizing the heterologous versus the homologous virus.

Also as shown in Table 1, rabbits were immunized with either strain of HMPV and then boosted with the same strain, and post-primary immunization and post-boost sera were collected and tested for the ability to neutralize the homologous and heterolgous subgroup virus. The initial immunization of rabbits resulted in sera that had relatively low neutralizing titers against either strain of HMPV, with titers in the range of ≤4.3 log_2 to 8.4 log_2. With these primary sera, there was no consistent difference between the efficiency of neutralization of the homologous versus the heterologous strain. Following the boost, the titers were substantially higher, in the range of 6.9 log_2 to 11.4 log_2, and in most cases the neutralization titer against the homologous strain was greater than against the heterologous strain. However, the difference was relatively modest, ranging from either no difference to a difference of 2.6 log_2. For example, animal R030, which had been immunized and boosted with strain 75, had a titer of 11.4 log_2 against the homologous strain 75 and a titer of 9.9 log_2 against the heterologous strain 83, corresponding to a 1.5 log_2 difference. The other rabbit (R031) that was immunized and boosted with strain 75 yielded serum that neutralized the homologous strain 75 four times (2.0 log_2) more efficiently than the heterologous strain 83. In the converse situation, with animals (R032 and R033) that had been immunized and boosted with strain 83, the titers following

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the boost either showed no difference for the heterologous versus homologous strain (animal R03), or showed 0.5 $\log_2$ higher homologous versus heterologous titer (animal R033). Thus, out of the eight post-immunizaton and post-boost rabbit sera, one had a lower homologous versus heterologous titer, three had no difference, and four had a greater homologous versus heterologous titer, with the

5   greatest difference being 2.6 $\log_2$. This indicates a trend towards greater homologous versus heterologous reactivity, but the inconsistent nature of the pattern and the low magnitude is consistent with the interpretation that the two strain exhibit a very high degree of cross-neutralization *in vitro*. Thus, the high degree of antigenic relatedness observed with post-infection hamster sera was confirmed with rabbit sera both upon initial immunization and following a boost.

10   The high degree of antigenic cross-reactivity observed *in vitro* indicates that at least one major HMPV neutralization antigen or major epitope is conserved between the HMPV subgroups. The genetic analysis presented in the previous Example showed that, of the three HMPV surface proteins F, SH and G, the F glycoprotein was highly conserved between subgroups. In contrast, the SH and G glycoproteins were highly variable and neither protein had no more than three contiguous

15   conserved amino acid in the extracellular domain, suggesting that conserved antigenic sites would be rare or nonexistent. The HMPV neutralization and protective antigens had yet been identified previously. In the case of RSV, the F and G surface proteins are the only significant neutralization antigens and the major protective antigens (Connors *et al.*, J. Virol. 65:1634-1637 1991), and in the case of the human PIVs the two surface proteins F and HN also are the neutralization and major

20   protective antigens. A subsequent Example (Example VI) will illustrate identification of a major HMPV neutralization and protective antigen effective in conferring protection against infection with either genetic subgroup.

Table 1: Homologous and heterologous HMPV antibody responses in rodents following immunization with HMPV strain CAN75 or CAN83

Rodent species and number	Immunizing virus[a,b]	Serum-neutralizing antibody titer[c] (recip. $\log_2$) against:	
		CAN75	CAN83
Hamsters			
2163	**CAN75**	8.9	6.9
2164	**CAN75**	8.3	5.3
2165	**CAN75**	7.9	5.9
2169	**CAN75**	·9.9	10.4
2170	**CAN75**	8.4	7.3
2166	**CAN75**	8.4	8.9
2167	**CAN75**	8.9	9.9
2168	**CAN75**	8.4	8.4
2173	**CAN75**	9.9	12.7
2174	**CAN75**	9.4	12.4
2175	**CAN75**	9.9	11.9
Hamsters			
2201	**CAN83**	7.3	10.7
2202	**CAN83**	8.4	11.3
2203	**CAN83**	10.9	11.3
2204	**CAN83**	7.9	10.3
2205	**CAN83**	7.9	10.3
2206	**CAN83**	8.9	11.7
2207	**CAN83**	7.9	10.3
2208	**CAN83**	8.9	11.7
2209	**CAN83**	9.9	12.3
2210	**CAN83**	8.9	10.3
Rabbits			
R030	**CAN75**	$\leq 4.3^{d}$	5.9^{d}
R030 (p.b.)	**CAN75**	11.4^{e}	9.9^{e}
R031	**CAN75**	$\leq 4.3^{d}$	$\leq 4.3^{d}$
R031 (p.b.)	**CAN75**	11.4^{e}	9.4^{e}
Rabbits			
R032	**CAN83**	$\leq 4.3^{d}$	6.9^{d}
R032 (p.b.)	**CAN83**	6.9^{e}	6.9^{e}
R033	**CAN83**	8.4^{d}	8.4^{d}
R033 (p.b.)	**CAN83**	8.4^{e}	8.9^{e}

a. Hamsters were inoculated intranasally with 10^4 $TCID_{50}$ of the indicated strain of HMPV.

b. Rabbits were immunized parenterally with 10^5 $TCID_{50}$ on day 0 with the indicated strain of HMPV, and were boosted on day 28 with 10^7 $TCID_{50}$ of gradient-purified virus of the same strain of HMPV.

c. Hamster neutralizing antibody titer, day 44 post-immunization serum. All animals had pre-immunization neutralizing antibody titers of ≤ 1.0.

d. Rabbit neutralizing antibody titer, day 28 post-immunization. All animals had pre-immunization neutralizing antibody titers of ≤ 1.0.

e. Rabbit neutralizing antibody titer post-boost, day 70 following the initial immunization. All animals had pre-immunization neutralizing antibody titers of ≤ 1.0.

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p.b.: post-boost

Prevalence of HMPV Serum Antibodies in Chimpanzees

Thirty-one captive chimpanzees *(pan troglodytes)* were screened for the presence of serum neutralizing antibodies to HMPV strain 75 or 83, using an *in vitro* infectivity neutralization assay with HMPV strain 75 or strain 83 as the antigen, (Table 2). Nineteen animals (61%) were found to be seropositive for either one or both HMPV strains. Serum neutralizing antibody titers to HMPV strain 75 ranged from undetectable (≤ 1.0 $\log_2$) to ≥ 9.6 $\log_2$. Serum neutralizing antibody titers to HMPV strain 83 ranged from ≤ 1.0 $\log_2$ to 8.6 $\log_2$. Twelve animals (39%) did not have detectable HMPV-75 neutralizing titers: these 12 animals also were seronegative to strain 83. Four additional animals were seropositive to strain 75 but seronegative to strain 83. These four animals had very low neutralizing antibody titers ($2.6 - 4.0$ $\log_2$), and their specificity is provisional. The prevalence of HMPV antibodies in chimpanzees indicates that these animals can become readily infected with HMPV in captivity, most likely from their human handlers. It also is possible, and indeed likely, that there is animal-to-animal transmission. The ability of these animals to be infected by contact from humans or other animals is a strong indication of permissiveness to HMPV. The finding that very few animals were seropositive for only one of the two strains suggests that these strains are highly related antigenically in primates and likely represent only a single HMPV serotype.

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Table 2: Prevalence of HMPV-neutralizing antibodies in chimpanzee sera

Sample number	Chimpanzee ID No.	Serum neutralization titer (recip. log$_2$) to indicated HMPV strain[1]:	
		75	83
1	99A005	6.6	4.6
2	A0A001	7.0	5.6
3	A0A002	5.6	4.0
4	A1A005	1.0	≤1.0
5	A1A007	≤1.0	≤1.0
6	A1A001	2.6	3.6
7	A1A002	5.6	3.6
8	A1A003	3.6	≤1.0
9	A1A006	≤1.0	≤1.0
10	A0A006	4.0	≤1.0
11	A0A007	4.6	3.6
12	A0A005	2.6	≤1.0
13	A0A009	3.6	≤1.0
14	AOA003	7.6	4.6
15	99A004	7.6	6.6
16	A2A001	≤1.0	≤1.0
17	A2A004	≤1.0	≤1.0
18	A2A006	≤1.0	≤1.0
19	A2A009	≤1.0	≤1.0
20	A2A011	6.0	4.6
21	A2A013	6.6	4.0
22	A2A014	6.6	5.6
23	98A008	8.6	6.0
24	99A001	8.0	5.0
25	99A002	8.0	6.6
26	99A003	9.6	8.6
27	A1A010	≤1.0	≤1.0
28	A1A012	≤1.0	≤1.0
29	A1A013	≤1.0	≤1.0
30	A2A007	≤1.0	≤1.0
31	A2A008	≤1.0	≤1.0
Control serum (pre)2		≤1.0	≤1.0
Control serum (post)3		8.3	8.3

1. Numbers in parentheses are the titers expressed as the reciprocal dilution log$_2$.
2. pre-immune serum from hamsters before inoculation with both HMPV 75 and 83.
3. Post-immunization serum obtained from hamsters following inoculation with both HMPV 75 and 83.

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HMPV Infection of Chimpanzees and Monkeys: Virus Replication, Immunogenicity and Effect of Previous Exposure to HMPV

The ability of HMPV strains 75 and 83 to infect chimpanzees identified as seropositive or seronegative by the HMPV neutralization assay described above was evaluated. As shown in Table

5   3, eight animals were organized into four groups (two animals per group): group 1 was seronegative for strain 75 and 83. Group 2 and group 4 were seropositive for both strains. Group 3 was seronegative for 83 and had low or no detectable neutralization titers to strain 75. Groups 1 and 2 were administered strain 75, and groups 3 and 4 were administered strain 83. The animals were inoculated intranasally (IN) and intratracheally (IT) with 1 ml per site of 1 X $10^{5.2}$ TCID$_{50}$/ml of the

10   indicated virus. Administration was by both the intranasal (IN) and intratracheal (IT) routes to provide the opportunity for the virus to replicate at each site (Hall *et al.*, Virus Res. 22:173-84, 1992; Crow *et al.*, Vaccine 12:783-790, 1994). Animals were housed and specimens collected as previously described (Durbin *et al.*, J. Infect. Dis. 179:1345-51, 1999; Skiadopoulos *et al.*, Virology 260:125-35, 1999). The animals were observed clinically for 16 days post-inoculation.

15   Nasopharyngeal swabs were collected on days 1 to 10 and 12 post-infection and tracheal lavages were collected on days 2, 4, 6, 8, 10, and 12 post-infection. Each of the four seronegative animals (groups 1 and 3) had nasal discharge on days 7, 8, 9 and 10 post-infection. As a precautionary measure, groups 1 and 3 were started on penicillin on day 8, because of evidence of congestion and noise in the lungs. One of the animals in group 1 had thick mucus in the day 8 post-infection TL

20   sample and had decreased appetite. The seropositive animals (groups 2 and 4) showed no signs of illness throughout the course of the study. Thus, HMPV seronegative chimpanzees can develop illness following infection with the 75 and 83 HMPV strains demonstrating that these viruses are infectious and virulent in chimpanzees. Since the recombinant HMPV strain 83 virus differs from its biologically-derived parent by only four nucleotide differences involved in creating the *Nhe*I marker

25   in the M/F intergenic region (FIG. 7), it is also considered a "wild type" virus in terms of genomic and phenotypic characteristics. Importantly, animals that were seropositive to both strains were protected from disease following challenge with either strain. This appears to be the first observation demonstrating the feasibility of immunizing against HMPV: since natural exposure protects against disease, it is reasonable to expect immunization with an appropriate immunogenic composition

30   comprising a recombinant HMPV of the current disclosure will elicit a desired immune response as well. These results indicate that serum neutralizing antibody titers of 4.6 (animal AOA003) to 7.0 (animal AOA001) log$_{10}$ are sufficient to confer protection from HMPV disease. Results obtained with chimpanzees are particularly relevant given the close evolutionary relationship between chimpanzees and humans.

Table 3: Infection of chimpanzees with HMPV

Group	Chimpanzee ID No.	Serum neutralization titer to HMPV strain: 75	Serum neutralization titer to HMPV strain: 83	HMPV administered[1]	clinical signs (day post-infection) rhinorrhea	clinical signs (day post-infection) cough
1	A1A005	≤1.0	≤1.0	75	6,7,8,9,10,12	
	A1A007	≤1.0	≤1.0		7,8,9,10	
2	99A005	6.6	4.6	75		
	A0A001	7.0	5.6			
3	A1A006	≤1.0	≤1,0	83	7,8,9,10	
	A0A005	2.6	≤1.0		7,8,9,10	5
4	AOA003	7.6	4.6	83	'	
	99A004	7.6	6.6			

1. The animals were inoculated intranasally (IN) and intratracheally (IT) with 1 ml per site of $10^{5.2}$ $TCID_{50}$/ml of HMPV strain 75 or strain 83.

The virus titers in the nasopharyngeal swabs and tracheal lavages taken from the HMPV-infected chimpanzees described in Table 3 were determined by an end point dilution assay with viral infectivity detected by immunostaining. As shown in Table 4, the chimpanzees shed relatively low levels of virus. This was particularly noteworthy for the animals in group 1, which were seronegative for both strains, and group 3, which was seronegative for strain 83: animals even though all of the animals in these groups exhibited clinical disease signs in response to infection, the mean peak titers of virus recovered from the nasopharyngeal swabs ranged from 1.9 to 3.2 log_{10}, and the titers from the tracheal lavage were 1.8 to 2.0 log_{10}. The swabs and lavages from animals in groups 2 and 4, which were seropositive to HMPV, either lacked detectable HMPV or had a very low titer (1.5 log_{10}). Thus, the lack of disease signs correlated with a reduced or undetectable level of virus shedding. The apparent incongruity between the low level of HMPV shedding in the presence of disease symptoms might reflect difficulty in retrieving infectious virus from secretions, possibly due to inactivation by host immune factors or a high level of cell-association of HMPV in the epithelial cells lining the respiratory tract of the chimpanzees. Since the chimpanzee is so closely related to humans, this raises the possibility that detection of HMPV in human secretions might also be inefficient. The inability to detect substantial HMPV shedding was unexpected, since RSV is known to be labile and cell-associated *in vitro*, but nonetheless is shed from RSV infected chimpanzees presenting with RSV-associated illness at a level that is 100 to 1000-fold greater than was the case for HMPV-infected chimpanzees (Belshe *et al.*, J. Med. Virol. 1:157-162, 1977). Alternatively, the level of HMPV replication in chimpanzees may indeed be low, but infection may trigger immune- or chemokine-mediated pathology resulting in development of disease symptoms (Glass *et al.*, Curr. Opin. Allergy Clin. Immunol. 3:467-473).

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Serum samples taken four to six weeks post infection contained high levels of serum neutralizing antibodies for every experimental group, with no clear difference between animals that had been seronegative or seropositive prior to the experimental infection. The post-immunization titers ranged from 7.4 to 10.2 $\log_{10}$, considerably higher than the range of 4.6 to 7.0 $\log_{10}$ that was associated with protection from clinical disease. Interesting, in this small sample there was no evidence of antigenic differences between strains 75 and 83. Specifically, seronegative animals that were immunized with either strain (groups 1 and 3) developed antibodies with titers that were somewhat higher against strain 83 than against strain 83, irrespective of the immunizing virus.

The animals that were seronegative for the strain used in the primary infection were next challenged with the heterologous HMPV strain. As shown in Table 4, both groups of animals were completely protected from cross-challenge, indicating that previous infection by one HMPV lineage protects chimpanzees from subsequent infection by the heterologous lineage. Thus, in the experimental animal that most closely resembles the human, immunity induced by an HMPV strain representing either genetic subgroup protected against infection with an HMPV strain from the heterologous subgroup.

It also was of interest to investigate the replication and immunogenicity of HMPV 75 and 83 in monkeys, which are more plentiful, less expensive and easier to house than chimpanzees. Twenty-two rhesus macaques and twenty African green monkeys were screened for serum neutralizing antibodies to HMPV, and each animal was seronegative to HMPV (≤ 1.0 $\log_2$). This limited sampling suggested that these two species of monkeys are not readily infected with HMPV by exposure in captivity. Four animals of each species were infected and intratracheally with $10^{5.2}$ $TCID_{50}$ of either HMPV strain CAN75 or CAN83. Four animals of each species were infected and intratracheally with $10^{5.2}$ $TCID_{50}$ of either HMPV strain CAN75 or CAN83 at each site, and nasopharyngeal swab samples were taken at days 1 to 10 and 12 post-infection, and tracheal lavage samples were taken at days 2, 4, 6, 8, 10 and 12 post-infection (Table 5). The samples were assayed for virus titer by end point dilution. Illness did not develop in any of the monkeys. The infected rhesus monkeys had very low titers of virus, ranging from 0.9 to 2.6 $\log_{10}$ $TCID_{50}$ (Table 5). Furthermore, the post-immunization titers of serum neutralizing antibodies were also relatively low, ranging from 6.3 to 8.4 $\log_2$. Thus, rhesus monkeys support the replication of HMPV, but the level of replication is low.

Infected African green monkeys (Table 5) shed titers of HMPV ranging from 2.2 to 4.9 $\log_{10}$ $TCID_{50}$, and developed serum neutralizing antibody titers that ranged from 9.4 to 10.9 $\log_2$. The time course of HMPV replication in samples collected from the nasopharynx and the trachea of the African green monkeys is shown in FIG. 35, and indicates that virus shedding occurred over a broad window of more than six days. These data showed that African green monkeys are permissive for HMPV replication and, in particular, represent a suitable primate host in which to evaluate the replication and immunogenicity of rHMPV derivatives for use in immunogenic compositions. In particular, the highest titers of virus and antibody were obtained with strain 83, which is the one

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represented by recombinant HMPV of this current disclosure. In this regard, the ability to monitor HMPV replication up to almost 5 $\log_{10}$ with concomitant immunogenicity provides a wide window for assessing effects on replication and immunogenicity due to the incorporation of attenuating mutations.

5   Thirty days following the first infection, the African green monkeys were challenged with the heterologous HMPV strain (Table 5). Both groups of animals were completely protected from cross-challenge, indicating that previous infection by one HMPV lineage confers resistance to subsequent infection by the heterologous strain. These data indicate that African green monkeys represent a suitable primate host in which to evaluate the protective efficacy of HMPV immunogenic
10   compositions.

          The studies described above provide data on the primary antibody response to strain 75 or 83 in seronegative animals, and describe the ability of the resulting antibodies to neutralize the homologous versus heterologous subgroup virus. These data were analyzed statistically by the Archetti-Horsfall formula (Archetti and Horsfall, J. Exp. Med. 92:441-462, 1950) to determine the
15   degree of antigenic relatedness based on *in vitro* neutralization. Each species was calculated separately, showing that the percent relatedness of the two HMPV subgroups was 48%, 64% and 99%, respectively, based on the data from hamsters, Rhesus monkeys and African green monkeys, respectively. This compares with a value of 25% and 31% for the two subgroups of RSV, based on analysis of animal and human sera, respectively (Johnson *et al.* J. Virol. 61:3163-3166, 1987; Hendry
20   *et al.*, J. Infect. Dis. 157:640-647, 1988). These data indicate that the two HMPV genetic subgroups are highly related antigenically and do not represent distinct serotypes or, likely, not even significant antigenic subgroups. This high degree of relatedness for the two HMPV subgroups is based on *in vitro* neutralization, and thus needs to be taken with some caution, since cross-neutralization in vitro might be mediated by a single highly conserved antigen, whereas cross-protection in vivo might
25   require the contribution of more than a single antigen, or might be augmented by the inclusion of additional antigens. Nonetheless, the present findings provide the first indication that the genetic diversity of HMPV can readily be accommodated by an HMPV vaccine, and that such a vaccine might need to represent only a single HMPV strain.

TABLE 4: Level Of Replication, Immunogenicity And Cross-Protection Of HMPV Strains CAN75 And CAN83 In Chimpanzees

Group no.[a]	Virus administered	Serostatus at time of inoculation	Pre-immunization serum neutralization titer[d] to:		Level of virus replication[e] in:		Post-immunization (day 28) serum neutralization titer[d] to:		Level of challenge virus replication[f] in:	
			CAN75	CAN83	NP swab	TL	CAN75	CAN83	NP swab	TL
1	CAN75	negative[b]	≤1.0±0.0	≤1.0±0.0	1.9±0.4	2.0±0.8	8.7±0.3	10.2±0.3	≤0.5±0.0	≤0.5±0.0
2	CAN83	negative[b]	3.8±1.5	≤1.0±0.0	3.2±0.5	1.8±0.3	8.9±1.0	10.2±0.3	≤0.5±0.0	≤0.5±0.0
3	CAN75	positive[c]	6.8±0.2	5.1±0.5	1.5±0.0	≤0.5±0.0	7.4±0.5	9.2±0.2	nd	nd
4	CAN83	positive[c]	7.6±0.0	5.6±1.0	≤0.5±0.0	1.5±0.3	8.4±1.5	10.2±0.2	nd	nd

a. Animals in groups of two were inoculated IN and intratracheally (IT) with $10^{5.2}$ TCID$_{50}$ of the indicated virus in a 1 ml inoculum at each site.

b. Chimpanzees in this group were seronegative (mean antibody titer of < 1:4) for the homologous infecting HMPV strain at the start of the study.

c. Chimpanzees in this group were seropositive (mean antibody titer of > 1:20) for the homologous infecting HMPV strain at the start of the study.

d. Serum neutralization antibody titer to the indicated virus is expressed as the mean reciprocal log$_2$ ± standard error.

e. Level of virus replication is expressed as the mean of the peak virus titers (log$_{10}$ TCID$_{50}$/ml ± standard error) for the animals in each group irrespective of sampling day. The NP titers on day 0 were ≤0.5 log$_{10}$ TCID$_{50}$/ml. The lower limit of detection is 1.0 log$_{10}$ TCID$_{50}$/ml. A value of ≤ 0.5 log$_{10}$ TCID$_{50}$/ml is assigned to samples with no detectable virus.

f. On day 35, animals were challenged IN and IT with $10^{5.2}$ TCID$_{50}$ of the heterologous strain of HMPV in a 1 ml inoculum at each site. Level of virus replication is expressed as the mean of the peak virus titers (log$_{10}$ TCID$_{50}$/ml ± standard error) for the animals in each group irrespective of sampling day. The NP titers on day 0 were ≤ 0.5 log$_{10}$ TCID$_{50}$/ml. The lower limit of detection is 1.0 log$_{10}$ TCID$_{50}$/ml.

nd: not done

TABLE 5: Level Of Replication, Immunogenicity And Cross-Protection Of HMPV Strains CAN75 And CAN83 In Two Species Of Monkeys

Primate species	No. of animals	Virus inoculum[a]:	Level of virus replication[b] in:		Post-immunization (day 28) serum neutralization titer[c] to:		Percent antigenic relatedness[d]	Level of challenge virus replication[e] in:	
			NP swab	TL	CAN75	CAN83		NP swab	TL
Rhesus	4	CAN75	1.3 ± 0.1	0.9 ± 0.2	6.4 ± 0.6	7.2 ± 0.3	64	nd	nd
Rhesus	4	CAN83	1.6 ± 0.1	2.6 ± 0.3	6.3 ± 0.1	8.4 ± 0.3		nd	nd
African Green	4	CAN75	2.6 ± 0.5	3.2 ± 0.4	9.4 ± 0.3	10.8 ± 0.4	99	1.4 ± 0.1	0.8 ± 0.1
African Green	4	CAN83	2.2 ± 0.2	4.9 ± 0.1	9.5 ± 0.5	10.9 ± 0.4		0.6 ± 0.1	0.9 ± 0.2

a. Animals were inoculated IN and intratracheally (IT) with $10^{5.2}$ TCID$_{50}$ of the indicated virus in a 1 ml inoculum at each site.

b. The level of virus replication is expressed as the mean of the peak virus titers (log$_{10}$ TCID$_{50}$/ml ± standard error) for the animals in each group irrespective of sampling day. Virus titrations were performed on LLC-MK2 cells. The lower limit of detection is 1.0 log$_{10}$ TCID$_{50}$/ml. A value of ≤0.5 log$_{10}$ TCID$_{50}$/ml is assigned to samples with no detectable virus.

c. Mean serum neutralization antibody titer to the indicated HMPV strain is expressed as the mean reciprocal log$_2$ ± standard error. All animals were seronegative for both strains of HMPV at the start of the study (that is, day 0 reciprocal neutralization titer of ≤ 1.0 log$_2$).

d. Percent antigenic relatedness was calculated using the formula of Archetti and Horsfall.

e. On day 30, animals were challenged IN and IT with10$^{5.2}$ TCID$_{50}$ of the heterologous virus in a 1 ml inoculum at each site. Level of virus replication is expressed as the mean of the peak virus titers (log$_{10}$ TCID$_{50}$/ml ± standard error) for the animals in each group irrespective of sampling day. The lower limit of detection is 1.0 log$_{10}$ TCID$_{50}$/ml.

nd: not done

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Development of a small animal model of HMPV replication

The evaluation of HMPV and rHMPV derivatives is further facilitated by the availability of a small animal model for evaluating replication and immunogenicity *in vivo*. Hamsters were infected intranasally with 10^6 TCID$_{50}$ of either strain 83 or 75, and animals were sacrificed on days 3, 4, 5, 6 or 7 and the nasal turbinates and lungs were harvested and assayed for virus titer. As shown in Table 6, each virus replicated to a peak titer greater than 6 log$_{10}$ in the nasal turbinates and 3.6 to 4.4 log$_{10}$ in the lower respiratory tract. Thus, hamsters were permissive for both subgroup strains. As shown in Table 1 (and in Table 7), infection with strain 83 induced serum neutralizing antibody titers of 10.3 to 12.3 log$_{10}$ against the homologous strain, and infection with strain 75 induced titers of 7.9 to 9.9 log$_{10}$ against the homologous strain. The basis for the apparent lower immunogenicity of strain 75 is not known, although this finding will need to be confirmed in a further study in which virus replication and immunogenicity are measured for each strain in the same study. The present data show that hamsters are relatively permissive for HMPV replication and provide a convenient small animal model for evaluating HMPV replication and immunogenicity.

The hamster model was used to evaluate cross-protection between the two HMPV genetic subgroups, as had also been investigated in chimpanzees as shown in Table 4. Groups of hamsters were infected with either strain of HMPV or with L15 diluent as a negative control. After 6 weeks the hamsters were challenged with either strain of HMPV and the level of pulmonary challenge virus replication in the respiratory tract on day 4 was determined, as described above. As shown in Table 7, previous infection by either HMPV strain induced a high level of protection against the homologous and heterologous HMPV strain in the upper respiratory tract. The lower respiratory tract was completely protected from challenge with either strain. In addition, sera were collected 31 days following the first infection and assayed to determine the neutralizing titer against each strain (Table 7). The results were very similar to those shown previously in Table 1: infection with CAN83 induced antibodies that were modestly more effective in neutralizing the homologous versus heterologous strain, but antibodies induced by infection with CAN75 neutralized the homologous and heterologous strains with similar efficiencies. These cross-protection data and reciprocal-neutralization assays indicated that CAN83 and CAN75 are antigenically highly related.

TABLE 6: DAILY LEVEL OF REPLICATION OF HMPV STRAINS CAN75 AND CAN83 IN THE UPPER AND LOWER RESPIRATORY TRACT OF INFECTED HAMSTERS

Virus inoculum[a]	Tissue	Mean virus titer (log_{10} $TCID_{50}$/g ± S.E.) on day[b]:				
		3	4	5	6	7
HMPV-CAN83	Nasal turbinates	6.0 ± 0.5	5.7 ± 0.3	5.1 ± 0.3	3.7 ± 1.2	1.7 ± 0.5
	Lungs	3.6 ± 1.0	3.1 ± 0.3	3.0 ± 0.3	≤1.5 ± 0.0	≤1.5 ± 0.0
HMPV-CAN75	Nasal turbinates	6.6 ± 0.4	6.4 ± 0.4	6.4 ± 0.6	5.2 ± 0.5	4.2 ± 0.7
	Lungs	4.4 ± 0.6	4.3 ± 0.7	3.3 ± 0.2	≤1.5 ± 0.0	≤1.5 ± 0.0

[a]Hamsters in groups of 30 animals were inoculated intranasally with 10^6 $TCID_{50}$ of the indicated virus.

[b]Nasal turbinates and lung tissues from six animals from each group were harvested on days 3 through 7 post-infection. Virus present in tissue homogenates was quantified by serial dilution on LLC-MK2 monolayers at 32°C. Infected cultures were detected with hamster anti-HMPV antibodies and immunoperoxidase staining. Mean virus titer for each group of hamsters is expressed as log_{10} $TCID_{50}$/gram ± standard error, S.E.

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TABLE 7: Hamsters Infected With HMPV Strain CAN83 Or CAN75 Are Protected Against Challenge With The Homologous And Heterologous HMPV Strains

Animals immunized with[a]	Mean serum-neutralizing antibody titer[b] (reciprocal $\log_2$) against:		Mean virus titer ($\log_{10}$ TCID$_{50}$/g) in nasal turbinates of hamsters administered the indicated challenge virus[c]:	
	CAN75	CAN83	CAN75	CAN83
CAN83	9.2 ± 0.2	10.4 ± 0.3	2.0 ± 0.2	1.7 ± 0.2
CAN75	10.9 ± 0.2	7.7 ± 0.2	$\leq 1.5 \pm 0.0$	2.4 ± 0.5
L15 medium	$\leq 1.0 \pm 0.0$	$\leq 1.0 \pm 0.0$	5.3 ± 0.1	5.6 ± 0.2

a. Hamsters in groups of 12 were immunized by IN infection with $10^{5.5}$ TCID$_{50}$ of the indicated virus or mock-infected with L15 medium.

b. Sera were collected 2-3 days before and 31 days following the first infection, and the neutralizing titer against each of the two strains was determined. The pre-infection anti-HMPV serum titers were ≤ 1.0 (reciprocal $\log_2$) for all animals in the study.

c. On day 42, six hamsters from each group were challenged IN with $10^{5.5}$ TCID$_{50}$ of CAN83 and the remaining six were challenged with CAN75. Nasal turbinates and lungs were harvested four days later, and the virus titer was determined on LLC-MK2 cells. The mean virus titer for each group of hamsters is expressed as $\log_{10}$ TCID$_{50}$/gram of nasal turbinate ± standard error.

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Example 6

Construction of a Human Parainfluenza Type I Virus (HPIV1) that Induces Neutralizing Antibodies and Protective Efficacy Against Both HMPV Genetic Subgroups

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To examine the ability of the HMPV F protein alone to induce serum neutralizing antibodies and to confer protection against HMPV challenge, a recombinant HPIV1 expressing the CAN83 F protein (rHPIV1-F$_{83}$) was recovered *in vitro* from an HPIV1 antigenomic cDNA containing the CAN83 F protein ORF inserted upstream of the HPIV1 N gene and flanked by HPIV1 GS and GE transcription signals (FIG. 36A). Expression of the F protein was confirmed by indirect immunofluorescence of infected cells (FIG. 36 B). Thus, rHPIV1-F$_{83}$ would express all of the proteins of HPIV1 as well as the F protein of HMPV.

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Groups of hamsters were immunized with CAN83, wild type rHPIV1, rHPIV1-F$_{83}$ or with L15 medium, and 33 days later sera were collected and tested for the ability to neutralize the CAN75 and CAN83 strains. As shown in Table 8, sera from animals immunized with CAN83 or rHPIV1-F$_{83}$ efficiently neutralized both strains of virus. In each case, neutralization of the homologous CAN83 strain was somewhat more efficient than for the heterologous CAN75 strain. The mean neutralization titer of sera from rHPIV1-F$_{83}$-infected animals was 1.2 log$_2$ lower than that observed from CAN83-infected animals, which corresponds well with the lower titer of rHPIV1 replication in hamsters in this experiment compared to CAN83. Thus, when adjusted for the level of virus replication, the rHPIV1- F$_{83}$ vector, expressing only a single CAN83 antigen, was as efficient in inducing CAN83-neutralizing antibodies as CAN83 itself.

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Fifty days following immunization, the animals were challenged by intranasal infection with rHPIV1, CAN75 or CAN83. As shown in Table 8, animals previously infected with rHPIV1-F$_{83}$ had a 125-fold and 158-fold reduction in the upper respiratory tract in the level of replication of the CAN75 and CAN83 challenge virus, respectively. Animals were completely protected in the lower respiratory tract. The rHPIV1-F$_{83}$ virus also protected against HPIV1 challenge, indicating that this virus was able to protect against the three respiratory tract pathogens, namely CAN75, CAN83 and HPIV1. This method also can now be used to determine the relative contribution of each of the other HMPV proteins to the induction of neutralizing antibodies and resistance to challenge virus replication. Importantly, these findings identify the F protein of HMPV as a major neutralization and protective antigen. Furthermore, the F protein of the CAN83 strain induced neutralizing antibodies and protection that were effective against both CAN83 and CAN75. This highlights the importance of this specific antigen in developing vaccines effective against all strains of HMPV.

TABLE 8: A Recombinant HPIV1 Expressing The CAN83 F Protein (RHPIV1-F$_{83}$) Equally Protects Hamsters Against Challenge With HMPV Subgroup CAN75 And CAN83 As Well As Against HPIV1

Virus inoculum[a]	Mean serum neutralization titer ($\log_2$) to indicated virus[b]:			Mean level of replication of the indicated challenge virus in the nasal turbinates[c]		
	rHPIV1	CAN75	CAN83	rHPIV1	CAN75	CAN83
L15 medium	≤1.0 ± 0.0	≤1.0 ± 0.0	≤1.0 ± 0.0	4.9 ± 0.3	5.0 ± 0.2	6.1 ± 0.3
CAN83	≤1.0 ± 0.0	8.3 ± 0.2	9.8 ± 0.2	4.6 ± 0.1	1.5 ± 0.0	1.5 ± 0.0
rHPIV1wt	6.1 ± 0.3	≤1.0 ± 0.0	≤1.0 ± 0.0	2.0 ± 0.2	4.4 ± 0.2[d]	5.8 ± 0.2[e]
rHPIV1-F$_{83}$	4.7 ± 0.2	6.0 ± 0.3	8.6 ± 0.2	1.9 ± 0.2	2.9 ± 0.3[d]	3.9 ± 0.1[e]

a. Groups of 18 hamsters were infected IN with 10^5 TCID$_{50}$ of the indicated virus or with L15 medium as a negative control.

b. Thirty-three days following the first infection, sera were collected and assayed to determine the neutralization titers against HPIV1, CAN75 and CAN83. 50 days following the first infection, six hamsters from each group were challenged IN with 10^5 TCID$_{50}$ of the indicated virus and the nasal turbinates were harvested after four days.

c. Virus present in the tissue homogenates was quantified and is expressed as the mean $\log_{10}$TCID$_{50}$/g for each group ± standard error.

d. Statistically significant difference between indicated values; $p < 0.01$ (unpaired t-test).

e. Statistically significant difference between indicated values; $p < 0.0001$ (unpaired t-test)

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Example 7

Construction of HMPV Recombinant Virus Expressing Protective Antigens for Both Putative Antigenic Subgroups of HMPV

As described above, different strains of HMPV can exhibit genetic diversity, especially in
5   the SH and G proteins, and there appear to be two major genetic subgroups. The preceding Example identified the HMPV F protein as a major neutralization and protective antigen effective against both subgroups. Thus, it is a preferred antigen for including in an HMPV vaccine. The preceding Examples showed that it is possible to achieve a high degree of protective immunity against both genetic subgroups by infection with a single HMPV from either subgroup. Furthermore, the F protein
10   was identified as a major neutralization and protective antigen effective against both subgroups. However, it may be that optimal immunogenicity and protective efficacy will be achieved by including the SH or G protein from either or both subgroups, or by including F protein from both subgroups. In addition, the available information indicates that HMPV is a significant cause of serious respiratory tract disease early in life, approximately coincident with RSV and the PIVs. Thus,
15   an immunogenic composition against HMPV likely would be administered at approximately the same time in life (optionally in a combinatorial formulation or coordinate immunization protocol for infants, and certain adult subjects) as immunogenic compositions directed against RSV and the PIVs. Accordingly, certain aspects of the current disclosure provide for broad coverage of an HMPV immunogenic composition, yielding increased effectiveness against multiple, antigenically-distinct
20   strains. It is also advantageous in certain embodiments to decrease the number of separate viruses that must be administered: an immunogenic composition comprised of multiple separate viruses is more difficult to develop and formulate since each virus must be verified to be safe and immunogenic separately and in combination, and issues such as viral interference can complicate formulation. The methods of the current disclosure offer a flexibility and versatility in design of immunogenic
25   compositions that addressees these and related problems.

In one exemplary strategy, one or more ORFs each encoding a heterologous, immunogenic protein or antigenic determinant (for example, fragment or epitope) thereof can be engineered into transcription cassettes and inserted into the genome of HMPV. For example, as illustrated in FIG. 34, an ORF encoding the G, F or SH surface protein of HMPV strain 75 is engineered to be flanked
30   by GS and GE signals from strain 83, and is inserted into the HMPV strain 83 backbone. This results in a chimeric virus that contains an additional gene and expresses all of the genes of strain 83 as well as a surface protein antigen of strain 75. This chimeric virus therefore expresses antigens representing both HMPV antigenic subgroups. This addresses the need to broaden coverage of immunogenic compositions, as well as meeting the need to minimize the number of separate viruses
35   that must be included in such compositions. A preferred heterologous strain HMPV gene for insertion into an HMPV backbone would be the F gene, since F has been directly identified as a major HMPV neutralization and protective antigen. A second preferred gene is the G gene, since its encoded protein appears to be highly divergent between HMPV strains, exhibiting only 70% amino

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acid identity between strain 00-1 and 83 compared to 98% identify for the F protein. Similarly, the SH protein is a third preferred gene for insertion into chimeric virus, since it also is relatively divergent and exhibits 85% sequence identity between strains 00-1 and 83. The example illustrated in FIG. 34 employed the same transcription signals and genome insertion site that was used for

5   expression of GFP. However, the methods of the current disclosure allow other signals or insertion sites to be tested. Also, the Example of FIG. 34 involved an ORF from a heterologous strain of HMPV, but the same strategy can be applied to other heterologous ORFs, such as ones from RSV, HPIV1, 2 or 3, other heterologous viruses or pathogens, and other molecules such as immunomodulatory proteins such as granulocyte-macrophage colony stimulating factor. In this way,

10   an HMPV backbone can be used to create additional viruses bearing the surface proteins of heterologous, non-HMPVs. Chimeric viruses of the current disclosure can then be administered on their own, or can be combined with the original parent or other rHMPV to make a multi-virus or multivalent immunogenic composition.

<div align="center">

Example 8

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15   **Methods of Screening for Compounds that Inhibit HMPV**

The current disclosure demonstrates that expression of the N, P, L and M2-1 proteins alone are sufficient to direct HMPV transcription and RNA replication. This was documented in two ways. First, expression of these proteins was sufficient to direct transcription and RNA replication of a cDNA-encoded mini-replicon derived from the HMPV genome and carrying a reporter gene. Second,

20   expression of these proteins was sufficient to launch the rescue of complete infectious HMPV from a cDNA-encoded antigenome. The antigenome is by definition the complement of the genome and is not able to serve as the direct template for transcription. Hence, both RNA replication and transcription must have occurred under the direction of the supplied proteins in order for a productive infection to be launched, indicating that N, P, L, and M2-1 are sufficient to direct all phases of

25   HMPV RNA synthesis. Therefore, the current disclosure provides novel and readily practiced methods for screening, identifying, designing, and characterizing compounds that inhibit one or more activities of HMPV (for example, transcription, translation, growth, infectivity, virulence, immunogenicity, etc.)

In addition, the current disclosure provides new, facilitated methods of monitoring infection

30   by complete HMPV which provide for the screening, identification and design of antiviral compounds that act at any stage in the HMPV replicative cycle. The current disclosure also provides methods for identifying the viral targets of antiviral compounds, thereby providing for improved design of compounds effective in inhibiting HMPV.

Potential antiviral compounds effective against HMPV include monoclonal or polyclonal

35   antibodies obtained by biological or recombinant methods, peptides or protein fragments (Lambert *et al.*, Proc. Natl. Acad. Sci. USA 93:2186-2191, 1996), sugar derivatives, polyionic polymers, anti-sense RNA, double stranded RNAs capable of meditating RNA interference RNA (RNAi) (Bitko and Barik, BMC Mirco. 1:34-44, 2001), and small molecule compounds (De Clercq, Antimicrob. Agents

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7:193-202, 1996; Prince, Ex. Opin. Invest. Drugs 10:197-308, 2002). In general, the methods will apply to any antiviral compounds capable of inhibiting HMPV in solution, in cell extracts, in cell culture, and *in vivo*. Antiviral compounds can act, for example, by inhibiting attachment, penetration (fusion), RNA synthesis, and virion assembly, and in other cases their specific target of action may

5   not be fully elucidated (De Clercq, Antimicrob. Agents 7:193-202, 1996; Prince, Ex. Opin. Invest. Drugs 10:197-308, 2002). Antiviral agents also can act by enhancing the host immune response, such as by stimulating the interferon-mediated antiviral state (Player *et al.*, Proc. Natl. Acad. Sci. USA 95:8874-9, 1998). In addition, an antiviral agent can be combined with an anti-inflammatory agent to effect a further reduction in disease (Prince *et al.*, J. Infect. Dis. 182:1326-1330, 2000).

10   In one embodiment of the current disclosure, rHMPV-GFP is used to screen antiviral compounds. In one exemplary strategy, rHMPV-GFP is treated with a test compound or library of compounds (for example, a compound or library of compounds prospectively including one or more antiviral agents) and compared with mock-treated control rHMPV-GFP for the ability to infect cells. Following a period of absorption, the inoculum containing the potential antiviral compound can be

15   removed by washing if desired. Infectivity is typically assayed by observation or measurement of a detectable label or signal whose presence or level of expression or detection is correlated with one or more activities of HMPV, for example as indicated by the development of green fluorescence due to expression of the GFP marker gene by recombinant HMPV-GFP, a method that can be monitored in living cells without compromising sterility over the course of the infection. Alternatively, the

20   antiviral agent can be included in the medium overlay for the duration of the infection. Alternatively, infection can be initiated in the absence of the compound, which can be subsequently added. In addition to identifying compounds capable of inhibiting one or more activities of HMPV, these assays can give an indication of the mode of action of a compound, such as whether it acts by directly neutralizing virus, or inhibiting subsequent gene expression, or inhibiting viral spread. While GFP is

25   provided as an exemplary label or marker, a wide range of useful "reporter sequences" (for example, reporter genes) and other sequences that direct expression of detectable labels and markers are known in the art and can be readily employed as detectable "signal" agents whose presence, level of activity or expression correlate with one or more selected activities of HMPV. Exemplary alternative markers and/or labels in this context include, *inter alia*, chloramphenicol acetyl transferase,

30   luciferase, secreted alkaline phosphatase, and a large number of other known reporter sequences routinely used in the art. This provides the ability to design a reporter virus or subviral construct that will be useful in a variety of high throughput screening protocols.

               Within related aspects of the current disclosure, the availability of rHMPV variants that each lack one or more of the viral ORFs, as described above, provides particularly useful tools and

35   methods to identify possible viral targets of antiviral compounds. For example, if a compound inhibits the infectivity of complete HMPV, it will be tested (for example, for its efficacy and/or target gene or protein specificity) against a panel of rHMPV lacking various genes, such as the ΔSH, ΔG, ΔSH/G, and ΔM2-2 variants mentioned previously. An alternative and complementary method of

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identifying targets for an antiviral compound involves forcing the virus to grow in the presence of the compound (present at minimal or suboptimal inhibitory concentrations if necessary). Under these conditions, it is expected that a large panel of useful variants will emerge that have developed increased resistance to the subject inhibitory compound. Sequence analysis of such variants can

5   identify mutations that are potentially responsible for the increased resistance. By the methods of this current disclosure, mutations suspected of conferring increased resistance can be introduced individually or in combinations into rHMPV, and the resistant rHMPV expressing a convenient reporter gene such as GFP will provide yet additional tools and methods to facilitate other phases of HMPV control and antiviral development. For example, the introduction of mutations identified in

10 resistant viruses provides a method of directly confirming that they confer resistance and thus distinguishes them from adventitious mutation that occur during passage. This provides direct identification of viral genes involved in drug resistance, and is an important tool for further drug development. As another example, as noted above, HMPV-GFP can be used to assay quickly and accurately to detect antibodies to HMPV. This can be advantageous in studies to develop monoclonal

15 or polyclonal antibodies capable of neutralizing HMPV as immunoprophylaxic or therapeutic agents. This also has applications in epidemiologic studies for example to determine the serostatus of individuals in a population. Furthermore, it provides a facilitated method to monitor changes in the level of HMPV antibodies in experimental animals or clinical subjects, such as in response to experimental immunogenic compositions, or in situations were the efficacy of an antiviral compound

20 is being evaluated. Also, the expression of a reporter gene such as GFP provides a useful marker to detect and quantify HMPV infection and immunity in individuals, including experimental animals, thereby providing an assay for viral disease detection, tropism and/or to identify and evaluate additional antiviral agents.

In other embodiments of the current disclosure, rHMPV expressing a convenient reporter

25 gene can be readily employed within screening compositions and methods based on cell-free *in vitro* assays. For example, a nucleocapsid preparation can be made from cells infected with rHMPV expressing a reporter gene, such as alkaline phosphatase or some other sensitively-detected enzyme or marker. Replicate *in vitro* assays can be constructed and used to screen compounds for antiviral activity in a high throughput format designed to detect the expressed reporter gene. This can be

30 based on the expression of a convenient reporter gene or, alternatively, can be based on the detection of RNA representing the marker gene or one or more HMPV genes. For example, mRNA produced in such an *in vitro* reaction mixture can be captured *in situ* by matrix-bound oligodT and, following washing to remove the reaction mixture, the resulting hybrids can be detected by secondary hybridization with probes tagged with reporter enzymes or ligands. Such assays can be further

35 modified to specifically target, for example, products of RNA replication or read-through of gene junctions as a measure of altered transcription.

The identification of the N, P, L, and optionally, M2-1 as proteins useful and/or sufficient to direct HMPV transcription and RNA replication provides additional new methods for screening

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compounds for anti-viral activity. In one embodiment, these four proteins can be expressed intracellularly together with a mini-replicon that encodes a reporter gene, resulting in reconstituted HMPV transcription and RNA. This can be used to screen for compounds, particularly those that are effective against HMPV transcription, RNA replication, and nucleocapsid assembly and function.

5   Alternatively, the N, P, L, and M2-1 proteins can be expressed at high levels, using recombinant baculovirus, vaccinia virus, bacteria or any other suitable expression system, and purified to use in reconstituted assays such as *in vitro* RNA synthesis, protein-protein interactions, or protein-RNA interactions. Alternatively, the N, P, L and M2-1 proteins can be tagged recombinantly by the incorporation of, for example, a hexa-Histindine tag or an epitope recognized by a monoclonal

10   antibody, such as the well known FLAG tag (Nilsson *et al.*, Prot. Exp. Pur. 11:1-16, 1997). These provide the basis for purification of these components by affinity chromatography, facilitating the development of *in vitro* assays. In addition, when such tagged genes are expressed from the context of infection by recombinant HMPV, it provides a method of isolating and identifying possible cellular and viral binding partners. Such binding partners can be identified by a variety of methods.

15   For example, in some cases, binding partners will be present in sufficient quantity that they can be visualized by gel electrophoresis and identified by conventional methods of protein sequencing. Low abundance binding partners can be identified from complex mixtures by mass spectrometric methods coupled with trypsin cleavage and protein sequencing. The identification of viral and cellular binding partners provides additional information to guide the development of compounds that inhibit HMPV.

20   While this disclosure has been described with an emphasis upon preferred embodiments, it will be obvious to those of ordinary skill in the art that variations of the preferred embodiments may be used and it is intended that the disclosure may be practiced otherwise than as specifically described herein. Accordingly, this disclosure includes all modifications encompassed within the spirit and scope of the disclosure as defined by the claims below.

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CLAIMS

We claim:

5   1.   An isolated recombinant human metapneumovirus (rHMPV), comprising

a partial or complete, recombinant HMPV genome or antigenome comprising one or

more attenuating nucleotide modifications, and

a major nucleocapsid (N) protein, a nucleocapsid phosphoprotein (P), and a large

polymerase protein (L).

10   2.   The rHMPV of claim 1, wherein the rHMPV is replication competent.

3.   The rHMPV of claim 1, wherein the recombinant HMPV genome or antigenome

further comprises a detectable heterologous sequence encoding a polypeptide.

4.   The rHMPV of claim 3, wherein the detectable heterologous sequence encodes a

reporter.

15   5.   The rHMPV of claim 4, wherein the reporter comprises green fluorescent protein

(GFP).

6.   The rHMPV of claim 3, wherein the detectable heterologous sequence is operably

linked to HMPV transcription gene start and gene end signals.

7.   The rHMPV of claim 1, wherein the one or more attenuating nucleotide

20   modifications comprises a partial or complete deletion of one or more rHMPV SH, G, M2-1,

M2-2, or M2 ORFs or one or more nucleotide substitutions that reduces or ablates expression of

the one or more rHMPV SH, G, M2-1, M2-2, or M2 ORFs.

8.   The rHMPV of claim 7, wherein the one or more attenuating nucleotide

modifications comprises a partial or complete deletion of one or more of the rHMPV SH, G,

25   M2-1, M2-2, or M2 ORFs, such that a functional protein is not produced.

9.   The rHMPV of claim 7, wherein the one or more attenuating nucleotide

modifications comprises a partial or complete deletion of a rHMPV SH ORF, such that a wild

type SH protein is not produced.

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10. The rHMPV of claim 7, wherein the one or more attenuating nucleotide modifications comprises a partial or complete deletion of a SH ORF of SEQ ID NO: 1, such that a SH protein comprising a sequence set forth as SEQ ID NO: 5 is not produced.

11. The rHMPV of claim 7, wherein the one or more attenuating nucleotide modifications comprises a partial or complete deletion of a rHMPV G ORF, such that a wild type G protein is not produced.

12. The rHMPV of claim 7, wherein the one or more attenuating nucleotide modifications comprises a partial or complete deletion of a G ORF of SEQ ID NO: 1, such that a G protein comprising a sequence set forth as SEQ ID NO: 6 is not produced.

13. The rHMPV of claim 7, wherein the one or more attenuating nucleotide modifications comprises a partial or complete deletion of a rHMPV SH and G ORFs, such that a wild type SH protein and a wild type G protein are not produced.

14. The rHMPV of claim 7, wherein the one or more attenuating nucleotide modifications comprises a partial or complete deletion of a SH ORF and a G ORF of SEQ ID NO: 1.

15. The rHMPV of claim 7, wherein the one or more attenuating nucleotide modifications comprises one or more nucleotide substitutions that reduces or ablates expression of a rHMPV M2-2 ORF.

16. The rHMPV of claim 15, wherein the one or more nucleotide substitutions that reduces or ablates expression of the rHMPV M2-2 ORF comprises one or more nucleotide substitutions that ablates one or more potential translation initiation codons of the rHMPV M2-2 ORF or introduces one or more in-frame stop codons into the r HMPV M2-2 ORF.

17. The rHMPV of claim 16, wherein the one or more nucleotide substitutions comprises substitutions of one or more nucleic acids of a rHMPV sequence set forth as SEQ ID NO: 1.

18. The rHMPV of claim 7, wherein the one or more attenuating nucleotide modifications comprises a partial or complete deletion of a rHMPV M2-2 ORF, such that a wild type M2-2 protein is not produced.

19. The rHMPV of claim 7, wherein the one or more attenuating nucleotide modifications comprises a partial or complete deletion of a M2-2 ORF of SEQ ID NO: 1.

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20. The rHMPV of claim 7, wherein the one or more attenuating nucleotide modifications comprises one or more nucleotide substitutions that reduces or ablates expression of a rHMPV M2-1 ORF, such that a wild type M2-1 protein is not produced.

21. The rHMPV of claim 20, wherein the one or more nucleotide substitutions that reduces or ablates expression of the rHMPV M2-1 ORF comprises one or more nucleotide substitutions that ablates the translation initiation codon of the rHMPV M2-1 ORF and further ablates additional ATG triplets in each reading frame of the rHMPV M2-1 ORF.

22. The rHMPV of claim 21, wherein the one or more nucleotide substitutions comprises substitutions at one or more positions of SEQ ID NO: 1.

23. The rHMPV of claim 7, wherein the one or more attenuating nucleotide modifications comprises a partial or complete deletion of a rHMPV M2 ORF.

24. The rHMPV of claim 23, wherein the partial or complete deletion comprises a partial or complete deletion of the M2 ORF of SEQ ID NO: 1.

25. The rHMPV of claim 1, wherein the one or more attenuating nucleotide modifications produces at least one desired phenotypic change in the rHMPV, wherein the phenotypic change comprises at least one of a change in growth properties in cell culture, a change in growth properties or virulence in the upper or lower respiratory tract of a mammalian host, a change in viral plaque size, a change in sensitivity or adaptation to temperature, a change in cytopathic effect, a change in the efficiency of transcription or genome replication, a change in sensitivity to interferon, a change in the efficiency of expression of one or more genes, or a change in immunogenicity.

26. The rHMPV of claim 25, wherein the one or more attenuating nucleotide modifications produces a change in viral growth in the upper respiratory tract, lower respiratory tract, or both, such that viral growth is attenuated by about 50-100 fold or greater, compared to growth of the corresponding wild type HMPV strain.

27. The rHMPV of claim 1, wherein the one or more attenuating nucleotide modifications comprises one or more nucleotide substitutions that produce one or more amino acid substitutions in a M2-1 or a L protein in the rHMPV.

28. An isolated, replication competent recombinant human metapneumovirus (rHMPV), comprising a partial or complete, recombinant HMPV genome or antigenome, a

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major nucleocapsid (N) protein, a nucleocapsid phosphoprotein (P), and a large polymerase protein (L), wherein the genome or antigenome of the rHMPV is rearranged such that an order of one or more genes or genome segments in the recombinant HMPV genome or antigenome is altered as compared to a wild type HMPV.

5   29. The rHMPV of claim 28, wherein the order of a SH, G, or F gene or genome segment is altered in the rHMPV genome or antigenome.

30. The rHMPV of claim 28, wherein the rHMPV genome or antigenome comprises a SH gene or genome segment and a G gene or genome segment inserted after a M gene and before a F gene of the rHMPV genome or antigenome.

10   31. The rHMPV of claim 28, wherein the rHMPV genome or antigenome comprises at least two copies of a SH gene or genome segment and at least two copies of a G gene or genome segment inserted after a M gene and before a F gene of the rHMPV genome or antigenome.

32. The rHMPV of claim 28, wherein the rHMPV genome or antigenome comprises a F gene or genome segment inserted after a 3' leader sequence and before a N gene of the
15   rHMPV genome or antigenome.

33. The rHMPV of claim 28, wherein the rHMPV genome or antigenome comprises a G gene or genome segment inserted after a 3' leader sequence and before a N gene of the rHMPV genome or antigenome.

34. The rHMPV of claim 28, wherein the rHMPV genome or antigenome comprises a
20   F gene or genome segment and a G gene or genome segment inserted after a 3' leader sequence and before a N gene of the rHMPV genome or antigenome.

35. The rHMPV of claim 28, wherein the rHMPV genome or antigenome comprises a G gene or genome segment and a F gene or genome segment inserted after a 3' leader sequence and before a N gene of the rHMPV genome or antigenome.

25   36. The rHMPV of claim 1, wherein the one or more attenuating nucleotide modifications comprises inserting one or more additional copies of one or more rHMPV G or F genes or genome segments in the rHMPV genome or antigenome.

37. The rHMPV of claim 36, wherein the rHMPV genome or antigenome comprises one or more additional copies of a rHMPV G gene or genome segment, a F gene or genome

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segment, or both, inserted after a 3' leader sequence and before a N gene of the rHMPV genome or antigenome.

38. The rHMPV of claim 36, wherein the rHMPV genome or antigenome comprises a single additional copy of a rHMPV G gene or genome segment inserted after a 3' leader sequence and before a N gene of the rHMPV genome or antigenome.

39. The rHMPV of claim 36, wherein the rHMPV genome or antigenome comprises a single additional copy of a rHMPV F gene or genome segment inserted after a 3' leader sequence and before a N gene of the rHMPV genome or antigenome.

40. The rHMPV of claim 36, wherein the rHMPV genome or antigenome comprises one additional copy of the rHMPV G gene and one additional copy of the rHMPV F gene in the order G-F.

41. The rHMPV of claim 36, wherein the rHMPV genome or antigenome comprises one additional copy of the recombinant HMPV G gene and one additional copy of the recombinant HMPV F gene in the order F-G.

42. The rHMPV of claim 36, wherein the rHMPV genome or antigenome comprises one additional copy of a rHMPV G gene or genome segment and two additional copies of the rHMPV F gene or genome segment in the order G-F-F.

43. The rHMPV of claim 1, wherein the rHMPV genome or antigenome further comprises one or more heterologous genes or genome segments from a different paramyxovirus to form a chimeric recombinant HMPV genome or antigenome.

44. The rHMPV of claim 43, wherein the rHMPV genome or antigenome comprises one or more N, P, or M genes from a different paramyxovirus.

45. The rHMPV of claim 44, wherein the paramyxovirus comprises avian metapneumovirus.

50. The rHMPV of claim 1, wherein the rHMPV genome or antigenome further comprises one or more rHMPV genes or genome segments from a different subgroup of HMPV to form a chimeric recombinant HMPV genome or antigenome.

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51. An immunogenic composition comprising an immunogenically effective amount of any one of the isolated, replication competent recombinant human metapneumoviruses of claims 1-50 in a pharmaceutically acceptable carrier.

52. A method for inducing an immune response in a subject against human metapneumovirus, comprising administering to the subject a therapeutically effective amount of any one of the isolated, replication competent recombinant human metapneumoviruses of claims 1-50, thereby inducing an immune response in the subject against human metapneumovirus.

53. The method of claim 52, wherein the recombinant human metapneumovirus is administered in a dose of 10^3 to 10^7 PFU.

54. The method of claim 53, wherein the recombinant human metapneumovirus is administered to the upper respiratory tract.

55. The method of claim 52, wherein the recombinant human metapneumovirus is administered by spray, droplet or aerosol.

56. An isolated, replication competent recombinant virus comprising a paramyxovirus genome or antigenome and a major nucleocapsid (N) protein, a nucleocapsid phosphoprotein (P), a large polymerase protein (L), and one or more recombinant genes or genome segments from human metapneumovirus.

57. The recombinant virus of claim 56, comprising a human metapneumovirus F gene.

58. The recombinant virus of claim 56, wherein the virus is an influenza virus or a parainfluenza virus.

59. An expression vector comprising an operably linked transcriptional promoter, a partial or complete, recombinant human metapneumovirus (rHMPV) genome or antigenome, and a transcriptional terminator.

60. The expression vector of claim 59, wherein the rHMPV genome or antigenome comprises one or more attenuating nucleotide modifications.

61. A method of screening an antiviral compound for inhibition of a biological activity of a human metapneumovirus, comprising

providing a recombinant human metapneumovirus (rHMPV) comprising a major nucleocapsid (N) protein, a nucleocapsid phosphoprotein (P), a large polymerase protein (L),

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and a partial or complete, recombinant HMPV genome or antigenome modified to incorporate a detectable heterologous sequence encoding a polypeptide correlated with the biological activity upon expression of the heterologous sequence;

exposing a test sample comprising the rHMPV or a host cell amenable to infection by HMPV to a test compound or library of test compounds that prospectively includes one or more antiviral agents capable of inhibiting the biological activity of HMPV;

providing a control sample comprising the rHMPV or host cell under suitable control conditions in the absence of the test compound or library of test compounds; and

detecting heterologous sequence in the test and control samples to determine an increase or decrease of the biological activity in the test sample compared to the control sample to determine the presence or absence of the antiviral compound in the test sample.
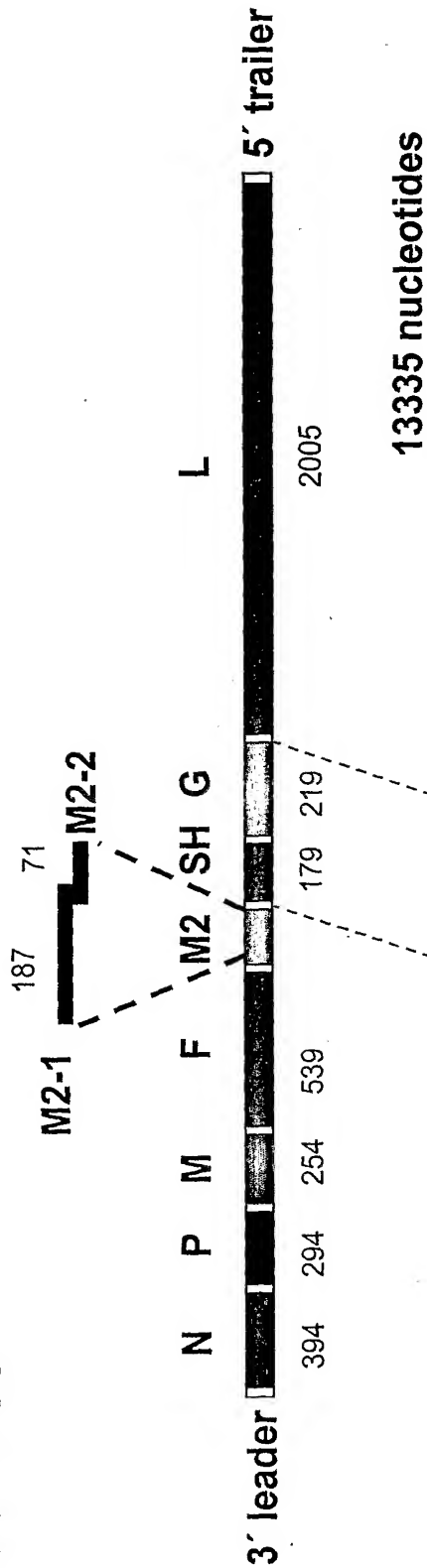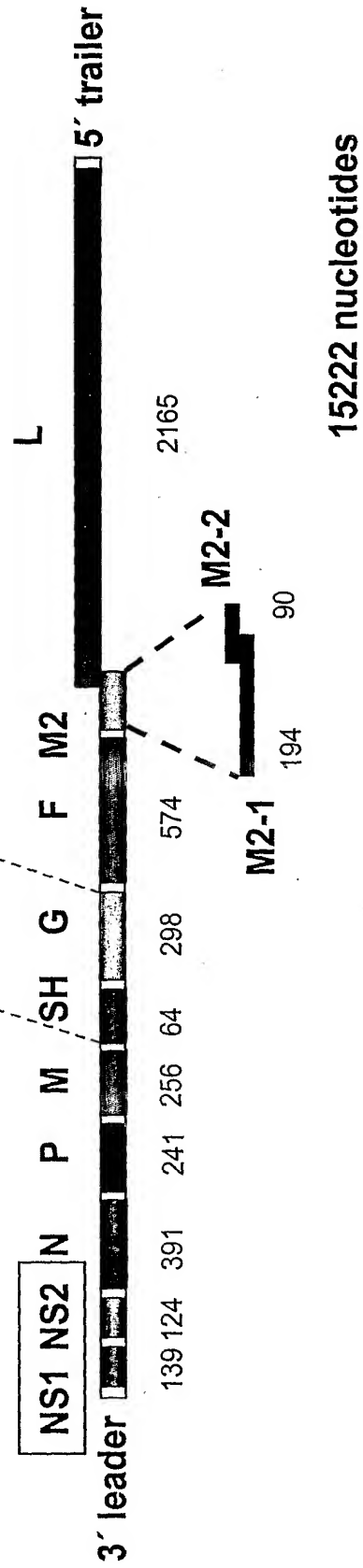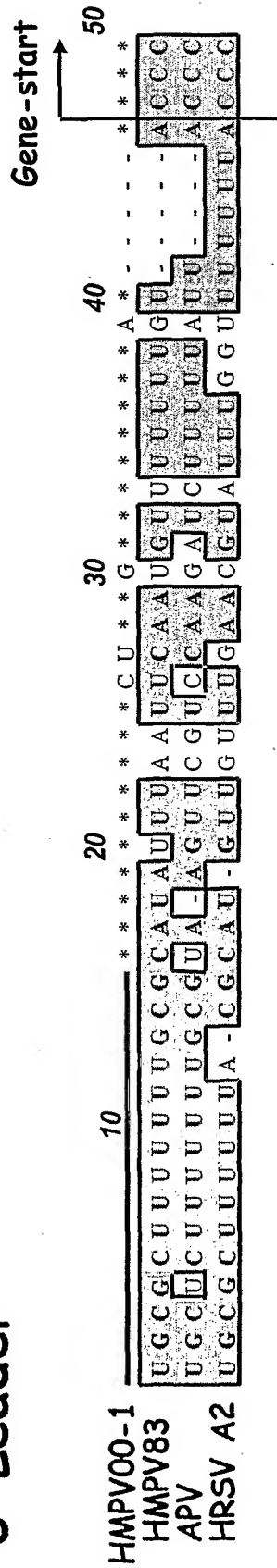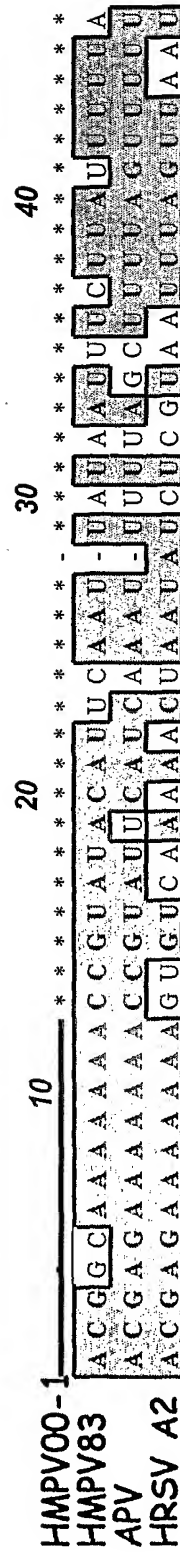
Fig. 1

Fig. 2

HMPV83 Le 3' UGGGCUUUUUGCGCAUAUUAAUUCAAUGUUUUUGUA
HMPV83 Tr 5' ACGGCAAAAAC-CGUAUACAUUCAUUAUUUCUUUAU

APV Le 3' UGCUCUUUUUG-CGUAAGUCGUCCAAGAUCUUUUUAUUA
APV Tr 5' ACGAGAAAAACCGUAUUCAUCUUAGCUUUUAGUU

Fig. 3

Examples of differences between HMPV 83 and HMPV 00-1
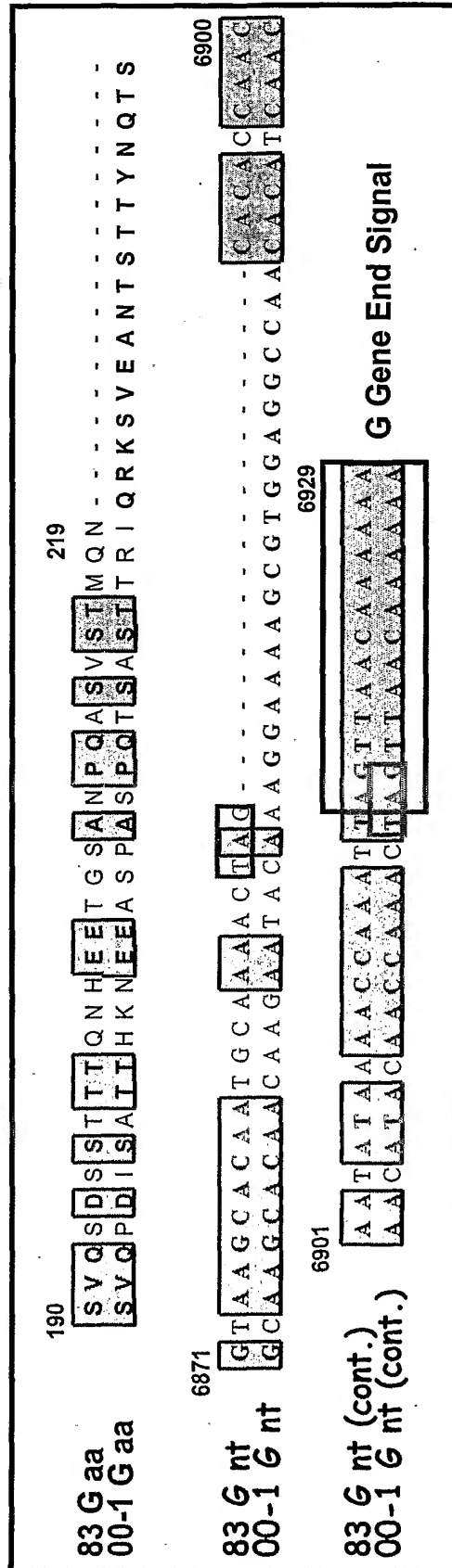
Fig. 4

Amino acid identity between HMPV83 and other Pneumoviruses for the indicated proteins

	N	P	M	F	M 2-1	M 2-2	G	SH	L
HMPV 00-1	99	95	99	98	98	96	70	85	99
HMPV 97-82	95	85	97	94	ND	ND	ND	ND	ND
APV A	70	58	77	68	73	25	12	20	64
APV B	69	53	76	67	71	27	ND	20	ND
APV C	88	68	87	81	83	56	ND	ND	ND
HRSV A2	41	31	38	36	36	12	15	6	46
HRSV B	41	31	37	35	35	8	15	6	46
BRSV	41	31	37	37	35	14	19	10	46
PVM	45	28	38	40	36	12	ND	8	ND

ND: Comparison not done, usually because sequence was unavailable

Fig. 5
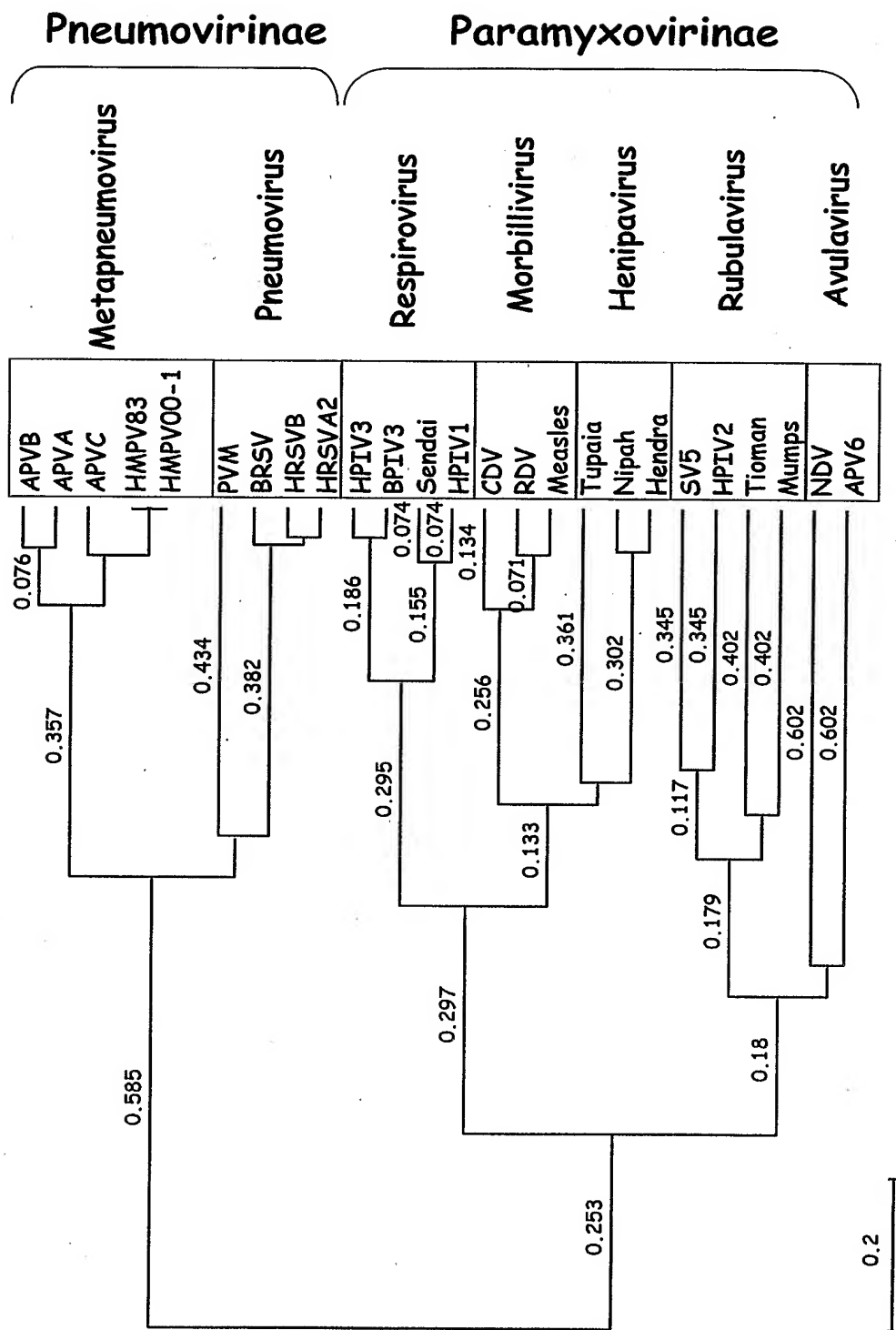
Fig. 6
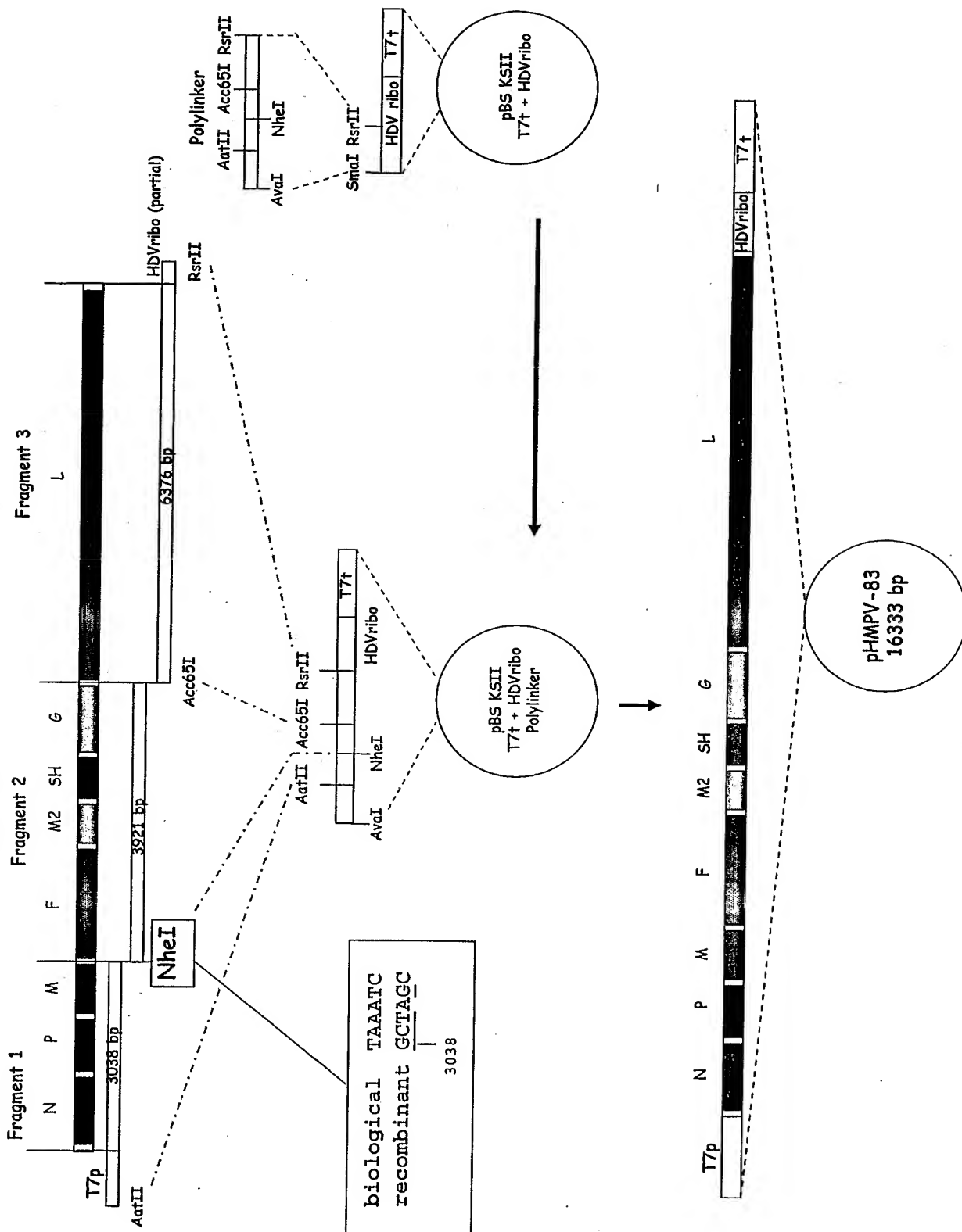
Fig. 7

Gene Start Signal

```
                          1           5         10        15
N     tacaaaaaacat  GGGACAAGTGAAA  ATGtctcttc
P     taattaaaaaagt  GGGACAAGTCAAA  ATGtcattcc
M     aaaaataaaaaat  GGGACAAGTGAAA  ATGgagtcct
F     atcaatcaagaac  GGGACAAATAAAA  ATGtcttgga
M2    taaaataaaattt  GGGACAAATCATA  ATGtctcgca
SH    aacacatcagagt  GGGATAAGTGACA  ATGataacat
G     aaaacaaaaatat  GGGACAAGTAGTT  ATGgaggtga
L     aaacagcatccaa  GAGACAAATAGCA  ATGgatcctc

CONSENSUS    ...at  GGGACAAGTGAAA  ATGtc...
                     gc   A  T  A AGTT     ga
                     ta          C C       at
```

Gene End Signal

```
                      1           5         10
N     ttatg  AGTAATTAAAAAA  gt
P     tatgt  AGTTTAATAAAAA  taaaaaat
M     atttt  AGTTATATAAAAA  tcaagttagaat  a
F     cagtt  AGTTAATTAAAAA  taaaataaaatt  t
M2    actta  AGTTAGTAAAAA   cacatcagagt
SH    agttt  AGTTATTTTAAAA  tatttgagaata  g
G     aaatt  AGTTAACAAAAAA  tacgagatagct  c
L     atgat  AGTTAATTAAAA   ttaaaaattaaaa  a

CONSENSUS    ...tt  AGTTAATTAAAAA  ta....
                    ag  ATTAAT  .  cc
                    ga      GC      gt
```

Fig. 8

Fig. 9

Fig. 10

Fig. 11

Fig. 12A

Fig. 12B

Fig. 13

Fig. 14

Fig. 15

Fig. 16

Fig. 17A

Fig. 17B

Fig. 17C

Fig. 18A

Fig. 18B

Fig. 18C

							C		X7		C		X5		C		X3	H	
HMPV-GFP M2 C7S	M S R K A P	C	[S]	K	Y	E	V	R	G	K	C	N	R	G	S	E	C	K	F	N	H	N
HMPV-GFP M2 Y9S	M S R K A P	C	K	Y	[S]	E	V	R	G	K	C	N	R	G	S	E	C	K	F	N	H	N
HMPV-GFP M2 C15S	M S R K A P	C	K	Y	E	V	R	G	K	[S]	N	R	G	S	E	C	K	F	N	H	N
HMPV-GFP M2 N16S	M S R K A P	C	K	Y	E	V	R	G	K	C	[S]	R	G	S	E	C	K	F	N	H	N
HMPV-GFP M2 H25S	M S R K A P	C	K	Y	E	V	R	G	K	C	N	R	G	S	E	C	K	F	N	[S]	N

Fig. 19

Fig. 20

```
                                                        4775
            4711                                GGC AAA TGC AAC AGA
            GGGACAAATCATAAATG  TCT CGC AAG GCT CCA TGC AAA TAT GAA GTG CGG
HMPV
rHMPV-GFP ΔM2-1                                            t  aa
                               tag         tag       t aaa
                               M   S   R   K   A   P   C   K   Y   E   V   R   G   K   C   N   R
HMPV
rHMPV-GFP ΔM2-1                 *           *         N   K              N              N
```

Fig. 21

Deletion of nt
4701 to 5459 (759 nts)
AAA/ATCAGAGT
AGTTAATTAAAAAcAc/gTacGAGTGGGATAAGTGACAATG
F gene end   intergenic   SH gene start

Fig. 22A

Fig. 22B

Reduction of virus yield in presence of exogenous interferon (IFN) type I

Virus	MOI	Fold reduction of virus yield		
		10 U IFN	100 U IFN	1000 U IFN
rHMPV-GFP	1.0	5	160	1680
rHMPV-GFP△M2(1+2)	1.0	19	1130	no virus
rHMPV-GFP△M2-2	1.0	13	250	no virus
RSV-GFP	0.01	4	17	90

Fig. 22C
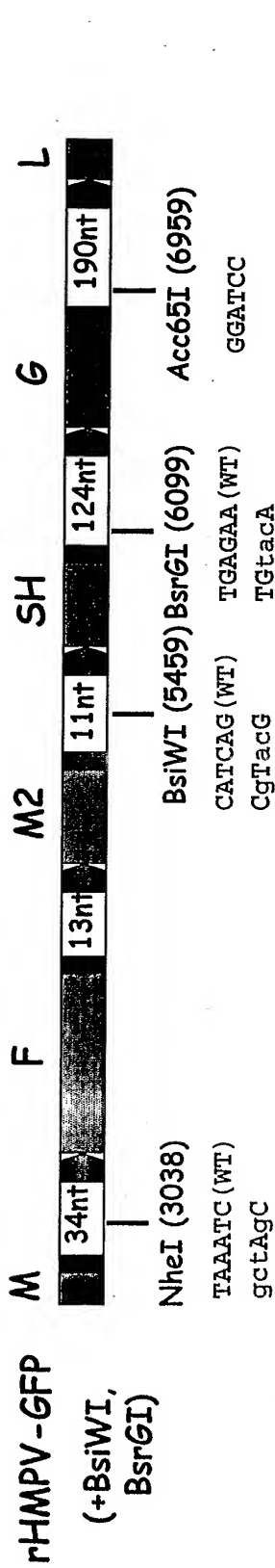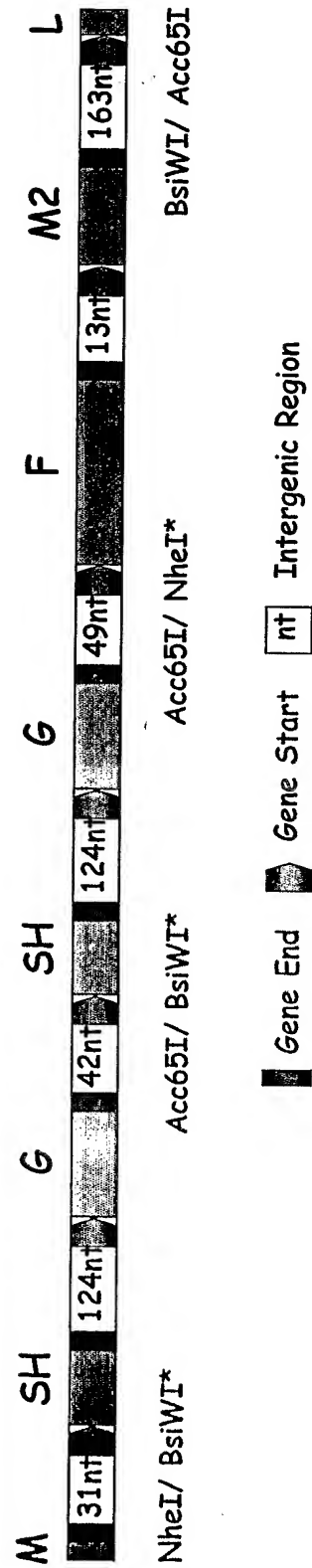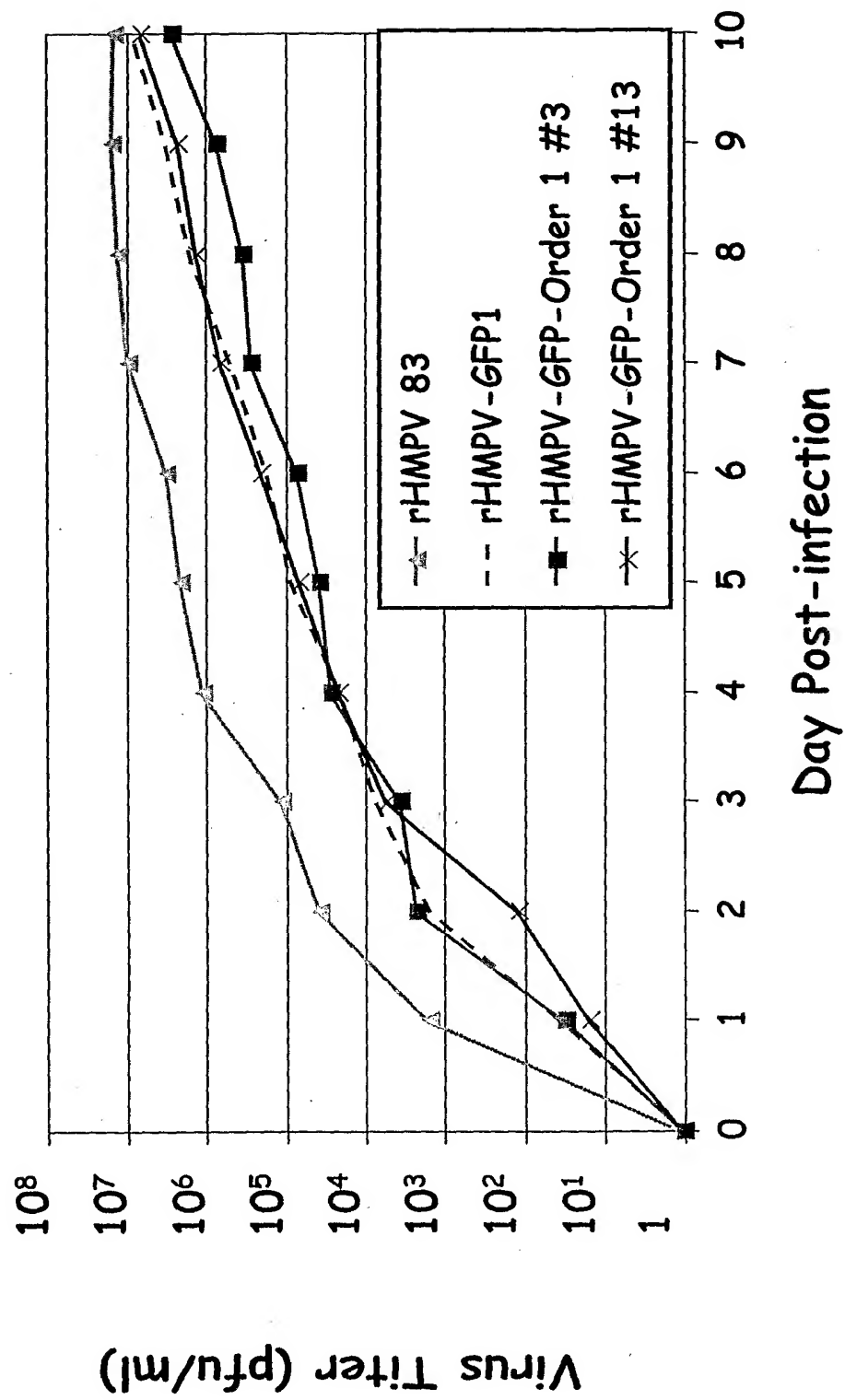
Fig. 23A

Fig. 23B
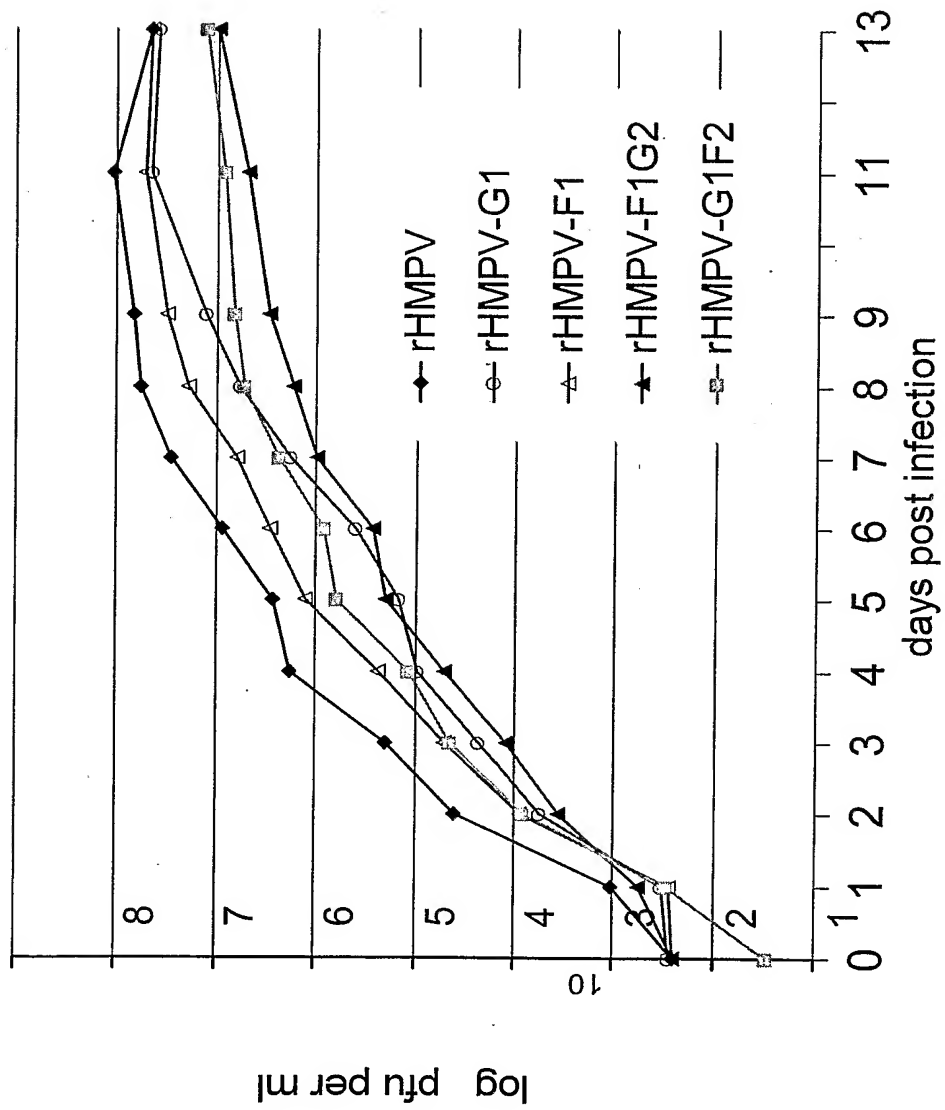
Fig. 24A

Fig. 24B
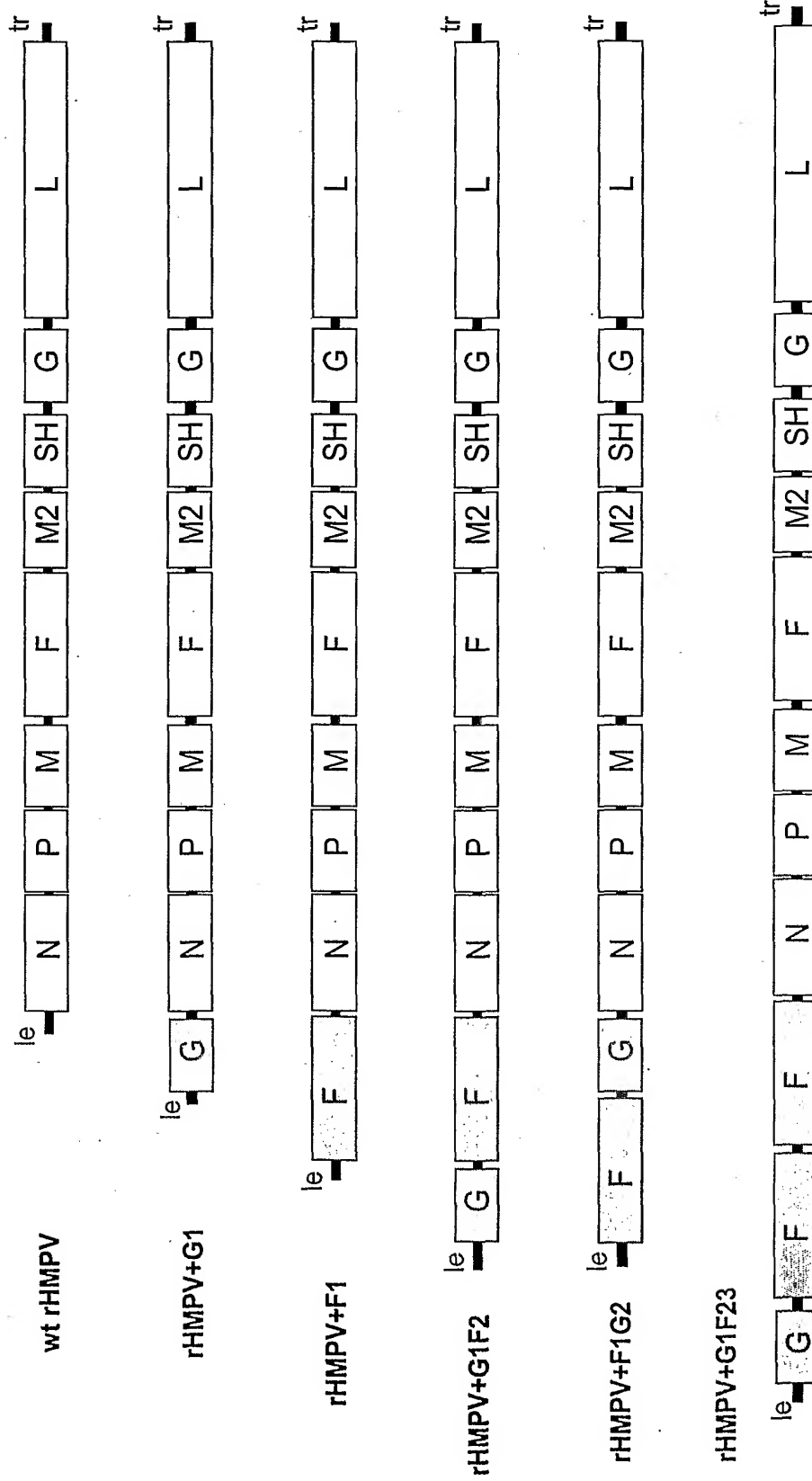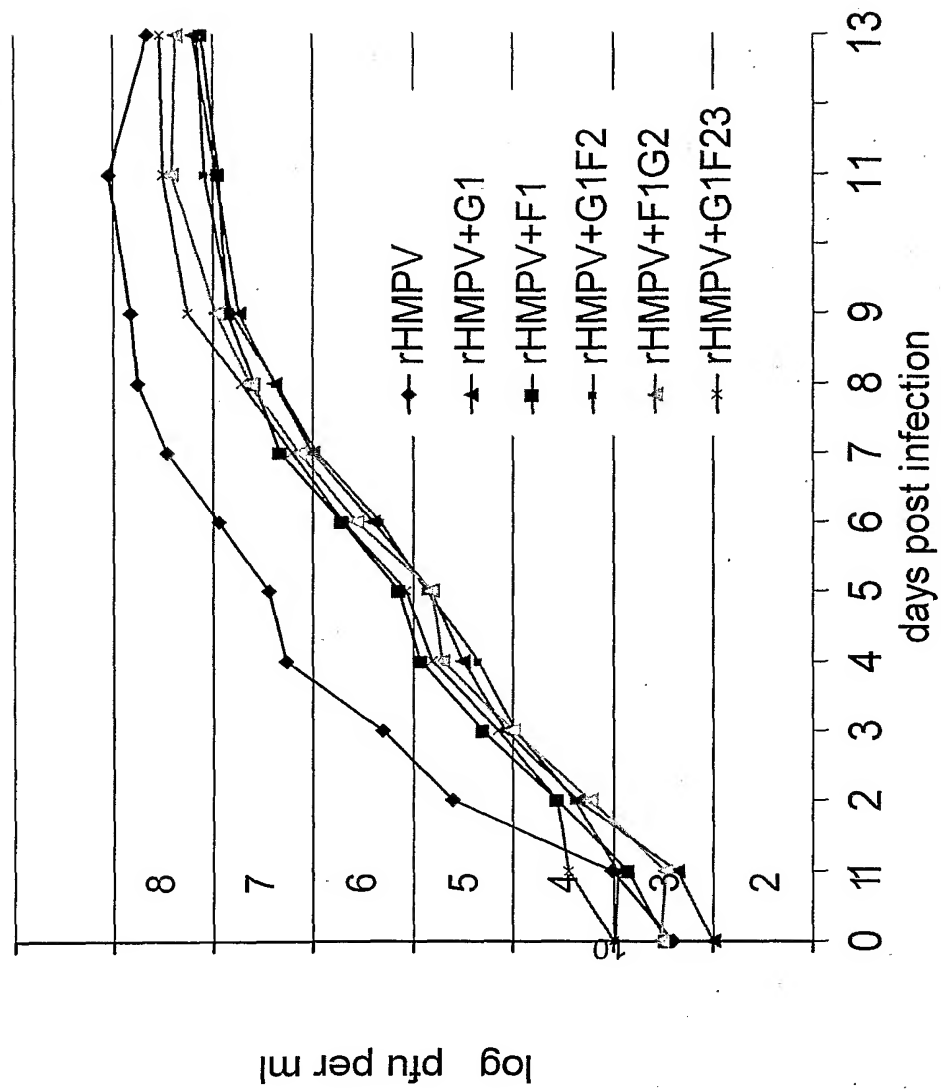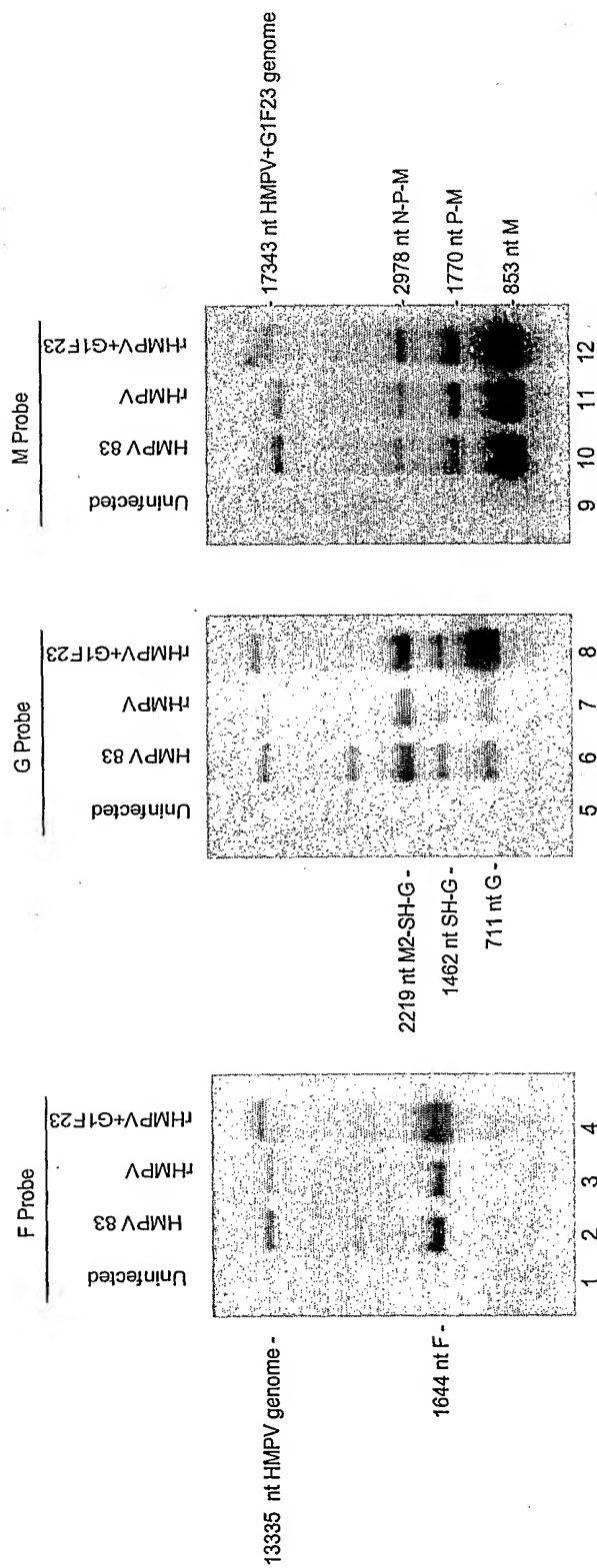
Fig. 25A

Fig. 25B

Fig. 25C

RSV A2 Mutations F521L and AA8

F521L (RSV)

hRSV A2	507	E L T E R D L I V L S G L R F Y R E F R L P K K V D L E M I N D K A I S P P K N L I W T - S F P R
hPIV3	442	E N A V D Y Y Q S F I G K F N K F I E P Q L D E D L T I Y M K D K A L S P K K - S N W D T V Y P A
hPIV1	442	E C A V D N Y S S F I G K F L K F I E P Q L D E D L T I Y M K D K A L S P R K - A W D S V Y P D
hMPV 83	442	E L S E Q D F L E L A A I Q F E Q E F S V P E K T N L E M V L N D K A I S P P K R L I W S - V Y P K
hMPV 001	442	E L S E Q D F L E L A A I Q F E Q E F S V P E K T N L E M V L N D K A I S P P K R L I W S - V Y P K

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hRSV A2	N Y M P S H I Q N Y I E H E K L K F S E S D K S R R V L E Y Y L R D N K F N	593
hPIV3	- - - - S N - - - - L L Y R T N A S N E S R R L V E V F I A D S K F D	517
hPIV1	- - - - S N - - - - L Y K V P E S E T R R L I E V F I N D N N F N	517
hMPV 83	N Y L P E T I K N R Y L E E T F N A S D S L K T R R V L E Y Y L K D N K F D	528
hMPV 001	N Y L P E K I K N R Y L E E T F N A S D S L K T R R V L E Y Y L K D N K F D	528

R588A
D589A } AA8 (double amino acid substitution)

Fig. 26A

HPIV3 Mutations Y942H and L992Y

Y942H

934

hPIV3	P	A	S	V	G	G	F	N	Y	M	A	M	S	R	C	F	V	R	N	I	G	D	-	-	P	S	V
hPIV1	P	A	N	I	G	G	F	N	Y	M	S	T	A	R	C	F	V	R	N	I	G	D	-	-	P	A	Y
hMPV 83	P	M	Q	F	G	G	D	P	V	V	F	Y	R	S	E	Y	R	S	E	Y	R	S	T	P	D	F	L
hMPV 001	P	M	Q	F	G	G	D	P	V	V	F	Y	R	S	F	Y	R	R	T	P	D	F	L

916

L992Y

hPIV3	Y	R	I	M	N	Q	E	P	G	E	-	S	S	F	L	D	W	A	S	D	P	Y	S	C
hPIV1	Y	R	V	M	N	Q	E	P	G	D	-	S	S	F	L	D	W	A	S	D	P	Y	S	C
hMPV 83	K	A	L	L	S	I	E	K	N	E	R	A	T	L	T	T	L	M	R	D	P	Q	A	V
hMPV 001	K	A	L	L	S	I	E	K	N	E	R	A	T	L	T	T	L	M	R	D	P	Q	A	V

996

1008
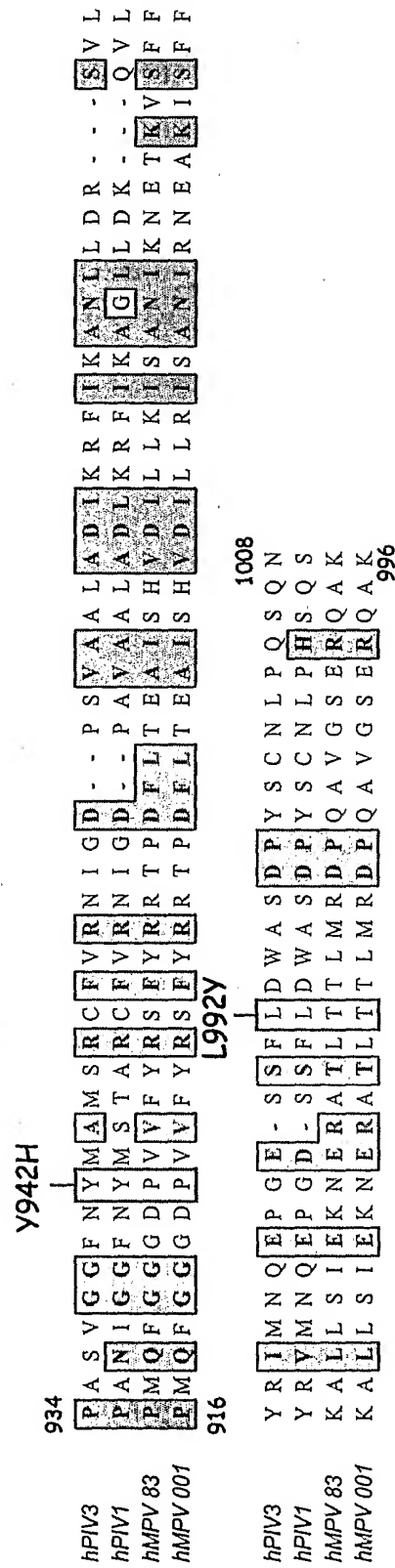
Fig. 26B

BPIV3 Mutation I1103V

I1103V

bPIV3	1087	R	V	G	I	S	R	G	-	G	L	T	Y	N	L	R	K	I	S	N	Y	D	L	V	Q	Y	E	T	L	S	1115
hPIV3	1087	R	V	G	I	N	R	G	-	G	L	T	Y	S	L	L	R	K	L	S	N	Y	D	L	V	Q	Y	E	T	L	S	1115
hPIV1	1087	R	A	S	V	R	R	G	-	G	L	S	Y	S	I	L	R	R	L	I	N	Y	D	L	L	Q	Y	E	T	L	T	1115
hRSV A2	1153	K	T	S	A	I	D	L	T	D	I	D	R	A	T	E	M	M	R	K	N	I	T	L	L	I	R	I	L	P	L	1182
hMPV 83	1078	R	T	S	A	I	N	G	E	D	I	D	R	A	V	S	M	M	L	E	N	L	G	I	L	S	R	I	L	S	V	1107
hMPV 001	1078	R	T	S	A	I	N	G	E	D	I	D	R	A	V	S	M	M	L	E	N	L	G	L	L	S	R	I	L	S	V	1107

HPIV3 Mutation T1558I

T1558I

hPIV3	1539	K	V	F	K	R	F	W	D	C	G	V	L	N	P	I	Y	G	P	N	T	A	s	Q	D	Q	I	K	L	A	L	S	I	C	E	1572
hPIV1	1539	K	I	F	K	R	F	W	D	A	G	V	V	E	P	V	Y	G	P	N	L	S	N	Q	D	K	I	L	A	I	T	S	V	C	E	1572
hRSV A2	1586	K	V	I	K	Y	I	L	S	Q	D	A	S	L	H	R	V	K	G	C	H	S	F	K	L	W	F	L	K	R	L	N	V	A	E	1619
hMPV 83	1507	K	V	K	K	R	I	M	L	Y	D	V	K	F	L	S	L	V	G	Y	I	G	F	K	N	W	F	I	E	Q	L	R	S	A	E	1540
hMPV 001	1507	K	V	K	K	R	I	M	L	Y	D	V	K	F	L	S	L	V	G	Y	I	G	F	K	N	W	F	I	E	Q	L	R	S	A	E	1540

RSV A2 Mutation C319Y

C319Y

hPIV3	258	K	L	Q	S	M	Y	Q	K	G	N	L	W	E	V	I	D	K	L	F	P	I	M	G	E	K	T	F	D	V	I	288
hPIV1	258	R	S	S	K	I	T	C	K	G	E	E	L	W	E	L	I	D	S	L	F	P	N	L	G	E	D	V	Y	N	I	I	288
hRSV A2	302	R	C	G	F	N	N	V	I	L	T	Q	L	F	Y	G	D	C	I	L	K	L	F	H	N	E	G	F	Y	I	I	332
hMPV 83	239	R	S	N	L	Q	G	M	L	T	N	K	L	Y	E	T	V	D	Y	M	L	S	L	C	C	N	E	G	F	S	L	Y	269
hMPV 001	239	R	S	N	L	Q	G	I	L	T	N	K	L	Y	E	T	V	D	Y	M	L	S	L	C	C	N	E	G	F	S	L	V	269

Fig. 26C

RSV A2 Mutation Q831L

Q831L

hPIV3	772	GDNQAIAVTTRVPNNYDYRIKKELVYKDVVRFFDSLR-EVMDDLGH	816
hPIV1	772	GDNQAIAVTSRVPVTQTYKQKTHVYEITRYFGALYKEYMFDLGH	816
hRSV A2	810	GDNQSIDISKPIRLMEGQT-HAQADYLALNSLKLYKEYAGIGH	853
hMPV 83	744	GDNQSIDVSKPVKLSEGLD-EVKADYRLAVKMLKEIRDAYRNIGH	787
hMPV 001	744	GDNQSIDVSKPVKLSEGLD-EVKADYSLAVKMLKEIRDAYRNIGH	787

RSV A2 Mutations M1169V, D1183E and C9

M1169V   D1183E

hPIV3	1087	RVGINRG-GLTYSLLRKISNYDLYQYETLSRTLR	1160
hPIV1	1087	RASVRRG-GLSYSILRLINYDLLQYLTRTLR	1160
hRSV A2	1153	KTSAIDLTDIDRATEMMRKNITLIRILPEDCNR	1222
hMPV 83	1078	RTSAINGEDIDRAVSMMLENLGLLSRILSVVDS	1147
hMPV 001	1078	RTSAINGEDIDRAVSMMLENLGLLSRILSVVDS	1147

hPIV3	LIYSDKIRYEDMCSVDLAIALRQKMWIHLSGGRMISGLETP	1160
hPIV1	KPVKDNIEYEYMCSVELAIGLRQKMWFHLTYGRPIHGLETP	1160
hRSV A2	DKREILSMENLSI-TELSKYVRERSLSN---IVGVTSP	1222
hMPV 83	TEIPIKSNGRLIC-CQISRTLRETSWNNME----IVGVTSP	1147
hMPV 001	IEIPTKSNGRLIC-CQISRTLRETSWNNME----IVGVTSP	1147

D1187A* R1189A*

K1188A* E1190A*

E1208A* R1209A*

* Six point mutations collectively designated C9

Fig. 26D

RSV A2 Mutation Y1321N

RSV A2 Mutation H1690Y

Fig. 26E

RSV A2 Mutation N43I

BPIV3 Mutation T1711I

Fig. 27

RSV

c cpts 248/404

```
          1    5   10   WT
M2    GGGGCAAAATA
```

HMPV

```
               1    5              15
N      t  GGGACAAGTGAAA ATG
P      t  GGGACAAGTCAAA ATG
M      t  GGGACAAGTGAAA ATG
F      c  GGGACAAATAAAA ATG
M2     t  GGGACAAATCATA ATG
SH     t  GGGATAAGTGACA ATG
G      t  GGGACAAGTAGTT ATG
L      a  GAGACAAATAGCA ATG

con. (exact)  t  GaGAcAAgTgaaa ATG
```

Fig. 28

Fig. 29

Fig. 30A

Fig. 30B-E

Fig. 31

gene-end

```
             1                                      13
le (83)  aattaAGTTAcaaAAAAAcat
   (75)  aattaAaTTccaaAcAAAAc-

N  (83)  ttatgAGTaAttaAAAAA--
   (75)  ttatgAGTaAttaAAAAA--

P  (83)  tatgtAGTTaatAAAAA--
   (75)  catgtAGTTtaatAAAAA--

M  (83)  attttAGTTAtatAAAAA--
   (75)  attatAGTTAtatAAAAAAA

F  (83)  cagttAGTTAattAAAAA--
   (75)  tagttAGTTAattAAAAAA-

M2 (83)  acttaAGTTAgtaAAAA---
   (75)  acttaAGTTAgtaAAAAA--

SH (83)  agtttAGTTAtttAAAAA--
   (75)  agtctAGTTAttAAAAA--

G  (83)  aaattAGTTAacaAAAAA--
   (75)  caagtAGTTAacaAAAAAAA

L  (83)  atgatAGTTAattAAAAA--
   (75)  ccattAGTTAattAAAAA--

consensus  AGTTAnnnAAAAA
```

intergenic

```
N:  gt
    ct

P:  taaaaaat
    taaacaat

M:  tcaag- 24 nt -agaac
    tttag- 20 nt -aaagc

F:  taaaataaattt
    t

M2: cacatcagagt
    taaatagaat

SH: tattt-114 nt -aatat
    ctcta-107 nt -aaaat

G:  tacga-180 nt -tccaa
    ctata-157 nt -ttcaa

L:  ttaaa-91 nt
    ttata-63 nt
```

gene-start

```
            1                           16
le (83)  GGGACAAgTgAaAAATgtctct    N (83)
   (75)  GGGACAAaTaAaAATgtctct        (75)

N  (83)  GGGACAAgTcAaAATgtcatt     P (83)
   (75)  GGGACAAgTcAaAATgtcatt        (75)

P  (83)  GGGACAAgTgAaAATggagtc     M (83)
   (75)  GGGACAAgTcAagATGgagtc        (75)

M  (83)  GGGACAAaTaAaAATgtcttg     F (83)
   (75)  GGGACAAgTaAaAATgtcttg        (75)

M2 (83)  GGGACAAaTcAtAAATgtctcg    M2 (83)
   (75)  GGGACAAaTcAtcATgtctcg        (75)

SH (83)  GGGAtAAgTgAcAATgataac     SH (83)
   (75)  GGGAtAAaTgAcAATGaaaac        (75)

G  (83)  GGGACAAgTagttATGgaggt     G (83)
   (75)  GGGACAAgTggccATGgaagc        (75)

L  (83)  GaGACAAaTagcAATGgatcc     L (83)
   (75)  GGGACAAaTaacAATGgatcc        (75)

                                   tr (83)
                                      (75)

consensus  GGGACAAnTnnnAATG
```

Percent amino acid or nucleotide sequence identity between the indicated strains of HMPV or RSV
for the indicated proteins and ORFs

Viruses compared	percent amino acid sequence identity for indicated protein (percent nucleotide sequence identity for indicated ORF)									
	N	P	M	F	M2-1	M2-2	SH	G	L
CAN97-83 vs CAN98-75	96 (84)	85 (81)	97 (85)	95 (84)	96 (85)	89 (87)	59 (69)	37 (59)	94 (84)
CAN97-83 vs 00-1	99 (94)	95 (92)	99 (94)	98 (94)	98 (94)	96 (95)	85 (88)	70 (77)	99 (95)
RSV A2 vs RSV B1	95 (85)	90 (85)	91 (85)	89 (81)	91 (84)	61 (69)	72 (77)	55 (67)	92 (85)

Fig. 32

Fig. 33A

B. G protein

```
          1    AR          F  KM   IRS   HR   T      S AP M    TL    DHA  54
CAN98-75  1    MEVKVENIRAIDMLKARVKNRVARSKCFKNASLILIGITTLSIALNIYLIINYT  54
CAN97-83  1                                     V                    K    54
00-1           **  . ***** .*** .**..*** .***  . .***.* .  *.*** ***

          55   TS  NMTKV  CVNM  V PS KTPMT  AAD NTK   P QA   LT   DS SLA T  108
CAN98-75  55   IQKTSSESEHHTSSPPTESNKEASTISTDNPDINPNSQHPTQQSTENPTLNPAA    108
CAN97-83  55   M  NT                          S  T SSP           GS  YF    108
00-1           *. .               *    **  . .          . ** .** * **.

          109  LEDHLH GTTP    A VSQQTT EH TLLRSTNRQ TQ TAEKKPTRATTKKET  162
CAN98-75  109  SVSPSETEPASTPDTTNRLSSVDRSTAQPSESRTKTKPTVHTRNNPSTASSTQS    162
CAN97-83  109  A SP    T      PPF  TH TP A     SA K    R S R H         162
00-1           *             .         * *.       *           . :

          163  TT T ST ATQTLNTTNQTSNG EA   AR RNNA   SSDQ TQ ADPS Q     216
CAN98-75  163  PPRATTKAIRRATTFRMSSTGKR-PTTTSVQSDSSTTQNHEETGSANPQASVS    215
CAN97-83  163       RTA  T L T   R  - S A   P I A   HKN  ASP S  T A     215
00-1           .*       .   .        .  .* .*.*        *.     .  * .* *

          217  QHTQKSTTTHNTDTSSPSS - 236
CAN98-75  217  QHTQKSTTTHNTDTSSPSS - 236
CAN97-83  216  TMQN------          219
00-1      216  TRIQRKSVEANTSTTYNQTS 236
               * .*.      .  ..
```

Fig. 33B

ORF encoding
G, F, or SH
of strain 75

Gene-start   Gene-end
(from HMPV 83 N gene)   (from HMPV 83 F gene)

GGGACAAGTGAAAATG   TAAGTTAATTAAAAAAGT IG

GGGACAAGTGAAAATGTCTCTTCAA

N Gene-start   N ORF

TTAAGTTACAAAAAAACAT

leader

41

HMPV 83 backbone
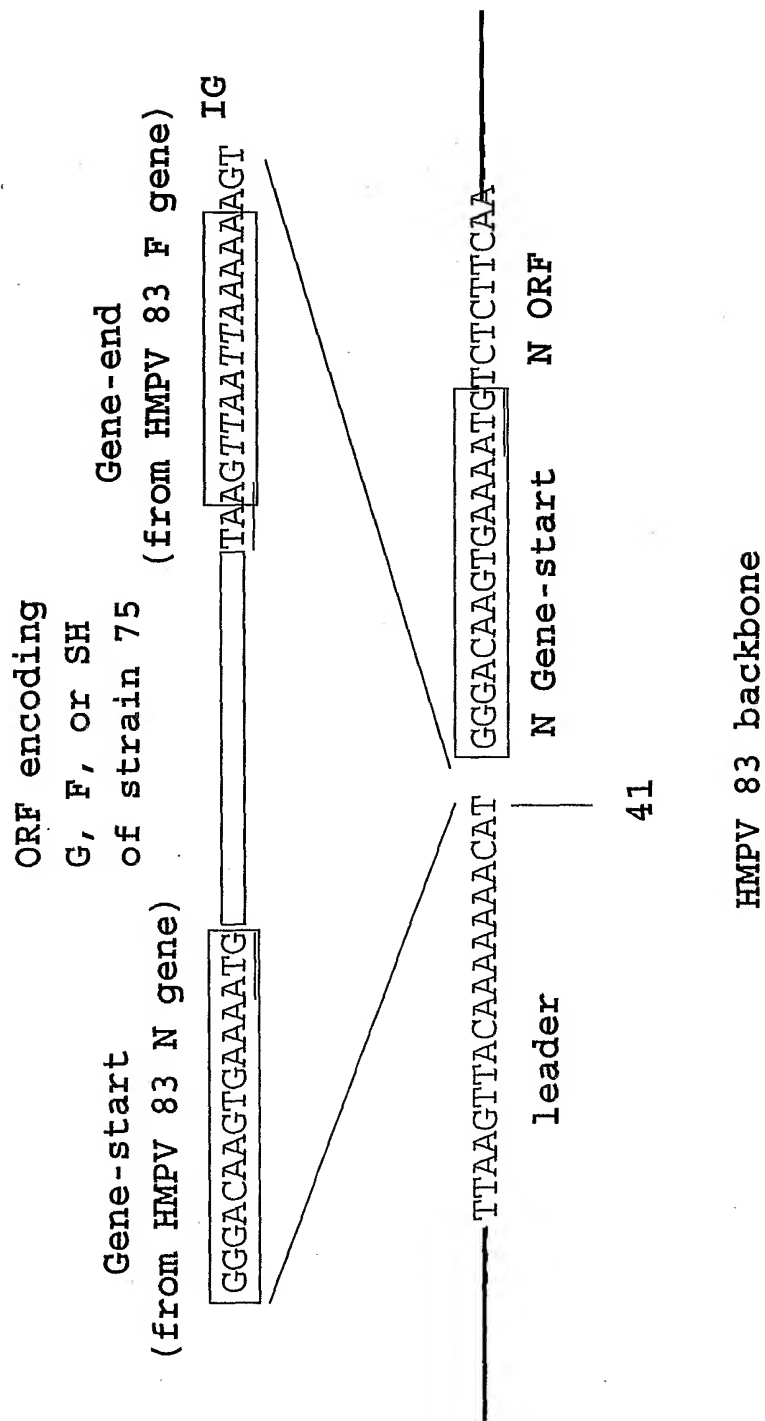
Fig. 34
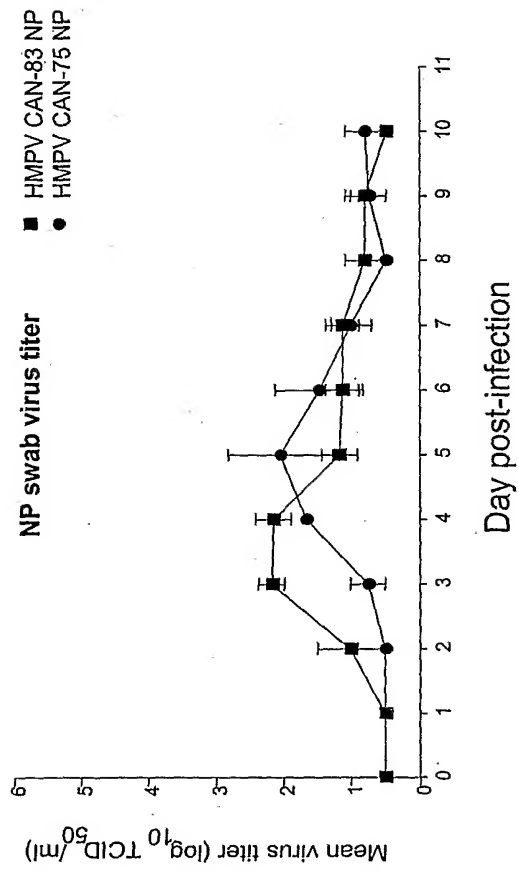
Fig. 35
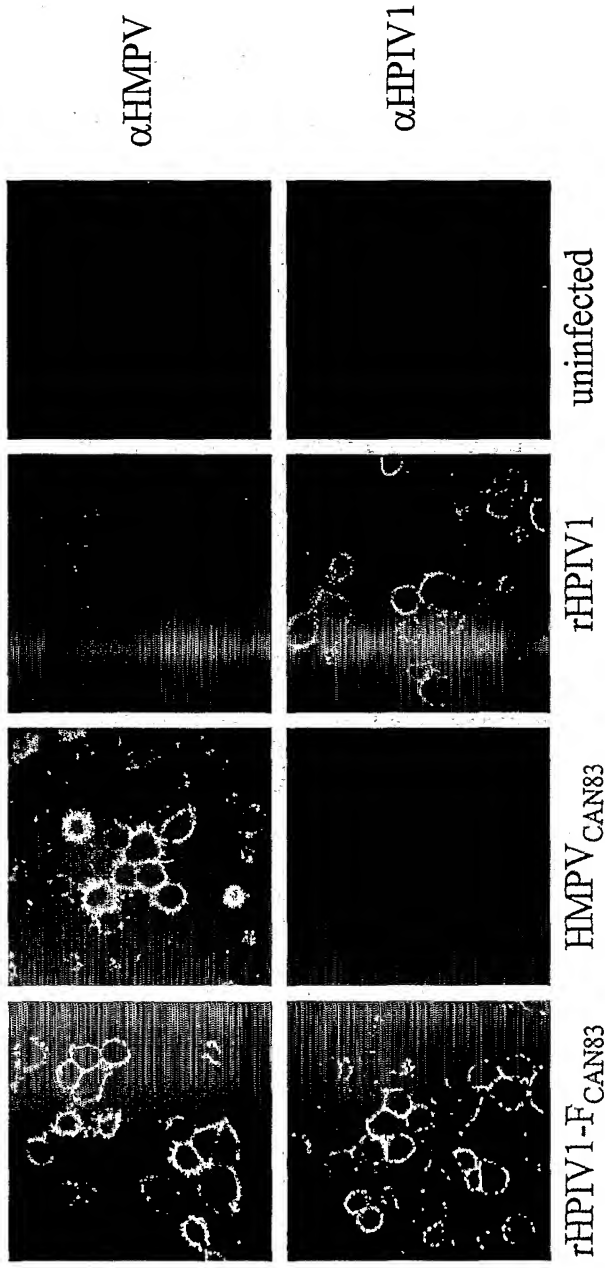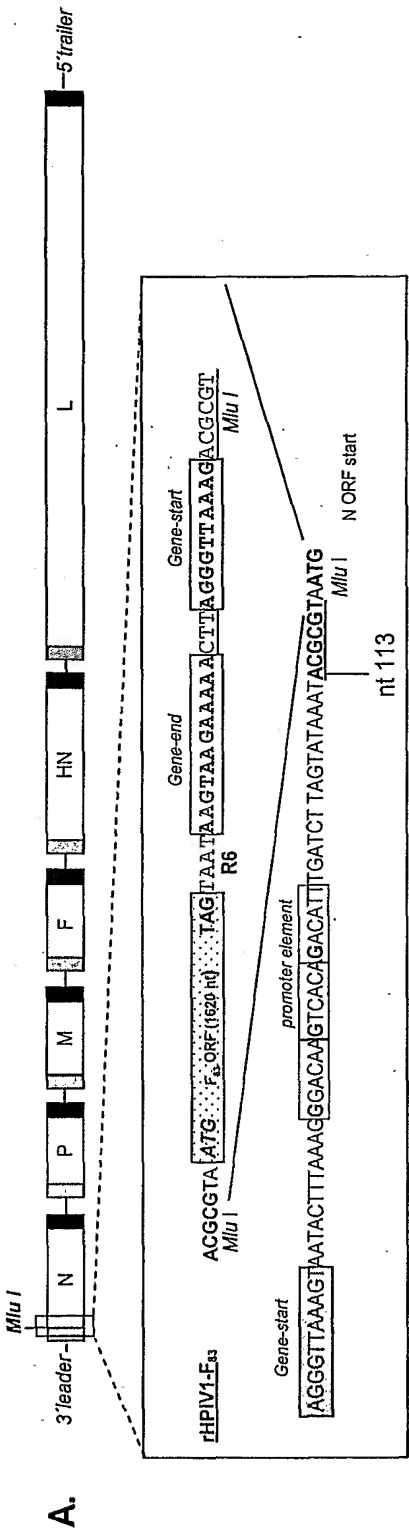
Fig. 36A & B

HMPV strain 83

```
           |10        |20        |30        |40        |50        |60        |70        |80        |90        |100
   1 ACGCGAAAAA AATTAAGTTA CAAAAAAACA TGGGACAAGT GAAAATGTCT CTTCAAGGGA TGATCTATCA TACAAGCATG                      100
 101 CTATATTAAA AGAGTCTCAG TATACAATAA AGGCACAACA ACAGCAGTGA CACCCTCATC ATTGCAACAA GAAATAACAC TATTGTGTGG          200
 201 AGAAATTCTA TATGCTAAGC ATGCTGATTA GCAAATATGCT GAATACAATA TATTAGCACA GCTCTAGGAT CAGAGAGAGT ACAGCAGATT          300
 301 CTTAAGAAACT AGTCCAGTGA GAACGTACTC GTTTTAACCA ACAAGGGAAA GTTAAAAACA ACAAGGAGAA GATTTACAG ATGTTAGACA           400
 401 TACACGGAGT AGAGAAAAGC TGGGTGGAAG AGATAGACAA AGAAGCAAGA ACAAGCAATA TAAAGAATCA TCAGGCAATA TTCCACAAAA          500
 501 TCAGAGGCCT TCAGCACCAG AGCTAACCTAT ACACACCTAT GCATGGCAAG CCTTAATATT TACCAAACTA TAGAAGTGGG ATTAGAGACC          600
 601 ACAGTCAGAA GAGCTAACCG TGTACTCAGT AATCTTATTA GATGCACTCA TAGGCAGCAA ATCATCAACTA AGCAGAGAAG TTTCTATGAT          700
 701 AAAAAGTGTA TTACAGAAGT TGTACTCATTG AAAGATACCC CAGGCAGCAA CAATATAATG TTATTCGTTA ATATATTCAT                      800
 801 GCAAGCTTAC GGTGCTGGTC AAACAATGCT GAGGTGGGGA GTCATTGCCA GTCATTGCCA AAAGCCCAAA AGCTGGACTG TTATCACTAG          900
 901 AAACAAGTCA CAGAAGTCTA TGACCTGGTG CGAGAAATGG GCCCTGAATC TGGGCTCCTA CATTTAAGGC TTATCGTCCA AGCTGAGTTA         1000
1001 CCAATTGTCC CAACTTTGCA AGTGTTGTTC TCGGCAATGC CTCAGGCTTA AAAGCCCAAA GTATGTATCG AACTATTTTC                     1100
1101 AGCAGCAGAA AGCTATGCCA AGAGTTTGAA AGAAAGCAAT TCAAATTAACT AAAATTAACT GATGAAGAAA AAGAGGCTGC AGAACACTTT         1200
1201 CTAAATGTGA GTGACGACAG TCAAAATGAT TATGAGTAAT CCAAAAATCA TTAAGAAAAG TTTTCATGGG GAACACTGTA                     1300
1301 TAATGAAGCG GCAAAATTGG CAGAAGCTTT TACTATCAGT TACTATTATAG AGAAAAAGT CATGGACAGA GCAATCAAAA                     1400
1401 TCTGAAACAT TGGAATTACC TACTATCAGT ATCCTATTGA AGAGTTAG CCGAAGTTAG CAAAGGTGGG CTCCTGCAGA                       1500
1501 CTGAAGCAAA GCAAACAATC AAAGAAGGTC TCATTTACAC TTTACTGAGA GCCCTCCAGT GATGGGGAAA CTCTAGACTG                     1600
1601 AAAGAAGTTG AAACCATCAA CCAATACTCAA AGGAAAATAC ACAAAGTTGG AGAAAGATGC TCTAGACTTG                               1700
1701 CTTTCAGACA ATGAAGAAGA AGATGCTCGA ACTTCATCAT AAGCATTGA AAGCCAGACTA GAATCGATTG                                1800
1801 AGGAGAAATT AAGCATGATA TTAGGGCTAT CAACATTGCT CCACAGCAGC ATCAGAGATG CAATGATGGG                                1900
1901 CATTAGGGAG GAACTAATAG CAGACATATT GATGGAAGAT AGCGGAAGAA AGCGGACAAA AATAGGAAAC                                2000
2001 GGTAGTGTAA AATTAACTGA AAAGGCAAAG AAAATTGTTGA ACAAGTGGTG ACAAGGAAGA CTAAAAGACA                                2100
2101 CACAGGAAAA TAATCAAGAA TAATCAGGA ACCAGTTAAT AAAAATGGGA CAAGTGAAAA TGGAGTCCTA                                 2200
2201 ACCTATCACC GCATCCCTTA CACAGCAGCT GTTCAAGTTG TTACCTGCAA TGGAGTTCCA CTGTTTCAGG                                2300
2301 CCAATACACC ACCAGCAGTT CTGCTTTGATC AGCTAAGAAC TCCCAAAAAG CCAATACTAA AAGTGAATGC                                2400
2401 ATCGGCCCAG GGTGCAGCAA TGTCTGTACT TCCCAAAAAG ATGCTGCATC AATTAGAATT TGACAAACTT                                2500
2501 ACAGTCTGTG AAGTAAAAAC AGTTTACTTA ACACCATGAA AAGTTTGTGA GCTCGGCCAA AAAAAAACAC                                2600
2601 ATGATCTAAT CGCATTATGG GATTTTATGG ATCTAGAAAA GTTCGGCCAA CAAATCAGTT TCTATCAAGG AGAGTGAATC                     2700
2701 AGCCACTGTT GAAGCTGCAA TAAGCTGCAA AAAGCTCATA TGCACCTTAT GCGGGACTGA TCATGATTAT GACCATGAAC                     2800
2801 AATCCCAAAG GCATATTCAA GAAGCTTGGA GCTGGGACCC AGCCTTATAT AGCGAGCAAA CATAAGGAAA ATATGGTAAC                     2900
2901 CTTGGAGCCA TCAAGGAACA ATACCAGGA ATAACAGCCA CCAAGAACTA CCAACTCTAT TCTATAGACT AAAAAAGTCGC                     3000
3001 CATTTTAGTT ATATAAAAAT CAAGGTTAGAA ATCAATCAAG AACGGGACAA CTTGGAAAGT GGTGATCATT TTTTCATTGC                    3100
3101 TAATAACACC ACATTAGAGG CTTCTGTACT CTTAAAGAGA TAAGAATTAA CTGAGGGATA TCTTAGTGTT CTGAGGACAG GTTGGTATAC         3200
3201 CAAGAGCTCA AGCTGCAGTC TGCTGACCAA TTGGCAAGAG GCTACCTAGA CCTAATAAAA ATCTGACCAA AAGTGCACTA                     3300
3301 TTGCAACAGC AGCTATGCC TTGGCAAGTC TTGCAATTGC AGAAAACCTT ACATGTTCTG AGACAATCTA GGTTTGTTCT AAACGACCAA           3400
3401 TGAAGCAGTA TCTACATTGG GGAATGGAGT CGAGTGTTG CAAAACCATC GGTTTGTTCT AATGCCCTCA GCACTCGGTG                      3500
3501 TGAAGGAGCT TCTACACGGT GGAATGGAGT ATGGCCGTTA ATGGCCAGAG TGAAAGACTT GTGAGCAAGA TGCAATCAAC                     3600
3601 AAAAACAAGT GCGACATTGA TGACCTAAAA ATGGCCGTTA GCTTCAGGTA ATGTTGTGCG AGGTTTCTAA GCAATCTGCA AATGATGTT           3700
3701 GAATAACACC AGCAATATCT TTGGACTTAA TGACAGATGC CTAACATGCC GACATCTGCA GGACAAATAA AATTGATGTT                     3800
```

Fig. 37A

HMPV strain 83 (continued)

```
3801 GGAGAACCGT GCGATGGTGC GAAGAAAGGG GTTCGGAATC CTGATAGGGG TCTACCGGAG CTCCGTAATT TACATGGTGC AGCTGCCAAT CTTTGGCGTT 3900
3901 ATAGACACGC CTTGCTGGAT AGTAAAAGCA GCCCCTTCTT GTTCCGGAAA AAAGGGAAAC TATGCTTGCC TCTTAAGAGA AGACCAAGGG TGGTATTGTC 4000
4001 AGAATGCAGG GTCAACTGTT TACTACCCAA ATGAGAAAGA CTGTGAAACA AGAGGAGACC ATGTCTTTTG CGACACAGCA GCGGGAATTA ATGTTGCTGA 4100
4101 GCAATCAAAG GAGTGCAACA TCAACATATC CACTACAAAT TACCCATGCA AGGAAGACAT CCTATCAGTA TGGTTGCACT GACAGCCAGC ATGTGGCACT 4200
4201 GGGGCTCTGG TTGCTTGCTA CAAGGAGTA  ACACTACACA TTGGCAGCAA CAGAGTAGGG GTTTATAAAA CTGCCGAGAG GCGCGAATTA AAAATTAGCA 4300
4301 ACCAAGATGC AGACACAGTG ATCAAGTTTC CTGAAGATCA TCAGGCTAAG AAGTTTGAGG GTGAACAGCA AACGCCAGG  CCTTGGTAGA TGTCAAGCAG 4400
4401 CTTTGATCCA ATCAAGTTTC CTGAAGATCA TCAATGTTT  ACTGGCTTCA TCAGGCTAAG AAGTTTTTGA GCTCTAGCAT GATCCTAGTG AGCATCTTCA 4500
4501 AGAATCCTAA GCAGTGCAGA GAAAGGGAAT AAACCAACCA AGCACCTCC  AGAGCTGAGT CATACCACAC AGTTAGTTAA TTAAAAATAA 4600
4601 TTATAATCAA GAAAACAAAG AAACCAACGG ATAATGTCTC GCAAGGCTCC ATGCAAAATAT GAAGTGCTT  ACAATGGCTT CATACCACAC TTAACCACAA 4700
4701 AATAAAATTT GGGACAAATC ATAATGTCTC GCAAGGCTCC ATGCAAAATAT GAAGTGCGGG GCAAATGCAA ACAATGGCTT AGTTAGTTAA TTAACCACAA 4800
4801 TTACTGGAGT TGGCCAGATA GATACTTATT AATAAGATCA GATTTTGTTC TAGGTTCCAC TTTAAGGAAC CAAAGCATA  ACTGATAGAG CTGATGGCCT ATCAATAATA 4900
4901 TCAGGCGCAG GCAGACGAGA CAGAACGCAA AGCAACTACA AGCAACTACA CTAGAGATAG TTGATGATAA CAAAGCATA  ACAGCAGC   ATGTGGCACT ACAAAAGCTG 5000
5001 CAGCCTGCTA CAGTCTACAC AACATAATCA ATCAATCA   GAGCAAAAACT CCCGCATCTT TCTCAAAGA CTGCCGAGAG AAAAATTAGCA AAAATTAGCA ATGTGGCACT 5100
5101 CCATAACTTA ATCTTATCTT ACATGGAGAT GACAATGACT CTTCATATGC CCTGCAAGAC AGTGAAAGCA TTAATCAAG  AAAAATTAGCA ATGTGGCACT AAAATTAGCA 5200
5201 AAGCTGATAA TTGACTTATC AGCAGGGCGT TGGAAATGAT A          GACAATGACT TCTCAAAGA  AGTGAAAGCA GCAGTGAGCA TGGTCCTGTT 5300
5301 TTCATTACTA TAGAGGTTGA TGAAATGATA ATTATATATG TGGACTCAAA AAGAATTAAA TAGTGAAGTC TCACACCAAC ATTTACAATT 5400
5401 GTTATTTAGA AAACATAGAA ATTATATAAT TCAAGGCTTA CTTAAGTTAG TCAGAGTGGG ATAAGTGACA ATGATAACAT TAGATGTCAT 5500
5501 TAAAAGTGAT GGGTCTTCAA AAACATGTAC TCACCTCAAA AAAATAATCA CTTATTGCAC CTTAAGTTAA TTAAGTTAAT ATTAGCTTTA 5600
5601 CTAACATTTT TCACAATAAC AATTACATAA AAGTAGAAAA AGTAGAAAA CAATCTACAA ATATGCCAGT ATCAGACAAA GAAGACTCAC 5700
5701 CATCAAATAC CACATCCGTC ACAACCAAGA CCACTCTAGA CCATCTTAGA TTAAAAGATT TTTATGCTTT AAACCTGAGG CTGTAATAAA 5800
5801 CAAGGACACA TGCTGGAAAA TAAGCAGAAA TCAATGCACA CAATATAACA AAACCTGAGG ATTGCATATA CAACAGTTGT CAACAGTTGT 5900
5901 GATAGACTGA CAGATCTATG CAGATCTATG ACCCAATCTT GAATGTTAAA ATTAATAAAC AGACTATCAG ATTGCATATA TGGAAGTGCT 6000
6001 ATCACCACTC AATAGATTAA ACCCAATCTT GAATGTTAAA GCTTAATTAT AATTAAAACA GATCCGTCTA ATATTAACA  AAAATATTTG 6100
6101 AGAATAGGTA AGTTTCTATG GACAAGTAGT TATGGAGGTG AAAGTAGAGA ACATTGAGC  TTAATTTACA TCGTGTGGCA TTAATTTACA 6200
6201 AAGTAAAAAC GCTTTAAAAA TGCTTCTTTA CACCACACCA GAATAAACTAC TCTATCTGAT CATAAACTAC TCGTGTGGCA ACAATACAAA 6300
6301 CGTAGCAAAT GCTTTCTTTA CCAACTCAAC AGTCCCCACA CTCAACCCCG AACAAGGAAG GCTTCAACAAT CCCTGATTCG AGCAATTTTC 6400
6401 AAACCTCATC TGAATCAGAA CACCACACCA AGTCCACACA CTCAACCCCG GAGCCCATCA AACCAGACA  TACTTAAAG  TCAATCCAAA 6500
6501 CTCACAGCAT CCAACTCAAC AGTCCCCACA AAACCCCACA CTCAACCCCG GAGCCCATCA AACCAGACA  CCAGACACA  ACCAGACACA 6600
6601 ACAAACCGCC TGTCCTCCGT ACAGCAGCCA CAACGAAGGC AATCGCAGA  CAGAACAGT  AAGAAACAAC CCAAGCACAC CCAAGCACAC 6700
6701 CTTCCAGTAC ACAATCCCCA ACAGGGGCAA CAACCAGCCA AATCATGAAA GCCACCACTT TCCGCATGAG ACAGGACAGA CCACAACATC 6800
6801 AGTCCAGTCC GACAACAGCA CAGATCTATG AAATCATGAA GAAAACAGTT CAGCAGACAA AAAGACCAA  GCACACCAAC GCACACCAAC 6900
6901 AATATAAAAC AACAATTAGT TACCAACCAA CAACATCACA CAACAACAT  AAGACAACT  ACCATCTCAA CACAATCTCC CACAATCTCC 7000
7001 CTAAAACAAC AACGACACCA CACAATTACA CCCTGATTCG AAAGTTGCCA TACTTAAAG  GAGTAATTTC GAAAAGAAAC GAAAAGAAAC 7100
7101 TGGGCAAAAT AGCATCCAAG AGACAAATAG CAATGGATCC TCTTAATGAA AATGGATATG ACTCTTGGGT ACCAACAGAT GAGTAATTTC 7200
7201 TTTTAGTGAA ACTAAATGCA TTGGTTCATG TCTCTTAAAA AGACCCTACT AAAGTTGCCA TGAGAATAC  TGTTATTGAG TGTTATTGAG 7300
7301 CATGTGAGAC TCAAAAATGA AGTCAATTCT AAAAATGAAAA TTTTAACAAG GAGCCAGTAA ACATGCAAGC TGAGAATAATG AAGAATGTAC 7400
7401 ACAGTTGTGA GCTCACACTA TTGATATATG AAATCATGAA TTTAACAAG  TATGATAATT GGTACAATGT AATTAAAGTC AATTAAAGTC 7500
7501 TACATCAGAT GATACCTCAA TCCTAAGTGT TTTAGTAATT GGTACAATGT TTTTATTGT  ATCATCATAT CAATAAGTTA CAATAAGTTA 7600
7601 ATTTTGGAAT TCAGAAGAGA GGAAGTAATA AGAACCGGTT TTTTATTGT  AAGCAACTGG TTTTATTGT  ATCATCATAT GGATGTATCG 7700
7701 TCAAGAGCAA CAAAAGCAAA AGAGTGAGCT TCTTCCACATA CAATCAACTG AAGATGTGAT TTTAATGCGA GTTAAGTAGA ATTTTGTAT  7800
```

Fig. 37B

HMPV strain 83 (continued)

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7801  ATGGGTAAGC AATAGTCTGA ATGAAAATCA GGAAGGGCTA GTAATCTGCA AGTTATGTTA ACTAATAAAC TATATGAAAC TGTAGATTAT  7900
7901  ATGCTAAGCT TATGTTGCAA TGAAGGTTTC TCACTTGTGA AGGTTTTATT ATGAGTGAGA TCCTTAGGAT TACTTGAACAT GCTCAATTCA  8000
8001  GTACTAGATT TAGAAATACT TTATTGAATG GATTAACAGA TCAATTAACA AAATTAAAAA ATAAAAACAG ACTCAGAGTT TATTAGAAAA  8100
8101  TAATGATTAT CCAATGTATG AAGTTGTACT TAAATTATTA GGAGATACTT CAAATTATTA ATCAATAAAA ACTTAGAGAA TGCTGCAGAA  8200
8201  TTATACTATA TATTCAGAAT TTTTGGTCAT CCAATGGTAG ATGAAAGAGA AATCACAAAA ATCCTAAGGT ACTTAAAAGT  8300
8301  TGGAGAGCTT GACAGAACTA AGAGGGGCAT TCATATTAAG GATTATCAAA AAGGTGGCCC AAAATTAAAA ACTTAAAAGT AATACAATTT  8400
8401  GCTTAGCAAA AGATGGACTA TGTACTTCAA AGCTAAAAAT TACCCCAGTC AAGTGAACAA GACTTTCTAG AGCTTGCTGC AATACAATTT  8500
8501  GAACAAGAGT TTTCTGTTCC TGAAAAAAACC AATCTTGAGA TGGTATTAAA CTAAAAGATT AATATGGTCT GTGTATCCAA  8600
8601  AGAATTACTT ACCTGAGACG ATAAAAAAAT GATATTTAGA AGAAACTTTC ATAGTCTCAA AACAAGAGA GTACTAGAGT ACTATTTAAA  8700
8701  AGATAATAAA TTTGATCAAA AGGAACTTAA AAGTTATGTA GTTAGACAAG CGATAAGGAG CACATTGTCT CATTAACTGG AAAAGAAAGA  8800
8801  GAATTAAGTG TAGGTAGAAT GTTTGCTATG CAACCAGGAA AACAGCGACA TTGGCAGAAA AATTGTTAGC TGATAACATT GTACCTTTCT  8900
8901  TCCCGGAAAC CTTAACAAAG TATGGTGATC TAGATCTTCA GAGAATAAATG CAGAACTTTC TTCTATCAAA ACCAGAAGAA ATGATAGTTA  9000
9001  TAATAATTAC ATTGCAAGAG AACAGATTTG AGCAAGTTCA ACCAAGCCTT TAGATATGAA TCTGTGCGGA TGTAGCAGAC  9100
9101  GAATTACATG GAACACAAAG CTTATTCTGT TGGTTACATC TATGACTACA ATGATATGTG TGCACCACCA GAAACAAAAG  9200
9201  GTGAATATGA TATAGATAAG ATAGAAGAGC AAAGTGGTCT CACATGGGCG GTATTGAAGG AAACTCTGGA CAATGGAAGC CAATGCTGGA  9300
9301  TATATCTTTA TGGGATGTTG TATCTGTAAA GACACGGTGT CGGTGACAAC CAATCAATAG ATGTAAGTAA ACCAGTCAAG  9400
9401  TTATCTGAAG GTTTAGATGA AGTGAAGGCA GATTATCGCT TTCAGTTTAT GAAAATAAGAG ATGCATACAG CATAAACTTA AGGTCTTGAG  9500
9501  AAGAAGGGGA AACATATATA TCAAGGGATC TTCAGTTTAA AAGCTAAATG CCTATCCACC AATTTAGGGG AGAAAGCATA  9600
9601  AGTAGGACCA TGGATAAAACA CAATATTAGA TGAGATTTTA CAATATTGGA GAGTCTATGT GAGAATTCTAA ACAGTTATTC AAACAACTAA  9700
9701  ATAGTTAGTC TGATATTAAG AAACTTCTGG CTGTATAACT TTGAAAATTAA ACAGTTATTC GAGGAGGAGA  9800
9801  ATAAAACATT AACATCAGTG TTCTCATTTT CAGAGATTTT TTGAAAATTAA AAGGGAAAAT ATGCAATTTG AGCTAACACA  9900
9901  TCCAGTAGTC TTCTATAGAT CTTTCTATAG AAGGACCCCT GATTTTTTAA CAATAGAAAA TAAAAATATC CAAGCTGTTG 10000
10001 AAAAATGAAA CGAAAGTAAG TTTTCTTCAA GCCTTACTAT CAATAGAAAA ACCAGTAGTT GAGAGATCCT ATAGTGCTAT 10100
10101 GATCAGAAACG ACAAGCAAAA GTAACAAGTG ACATCAATAG AACAGCAGTT TAAGTCTTTC CCCAAAATCAA CTTTTCAGTG ATTGCCCTTT 10200
10201 ACACTACAGC AGAAATGAAG AAGAAGTGGG AATCATTGCA GAAAACATAA TCCTCATGGG CTGAGAGTAT TATATGAATC ATTGCACAGG 10300
10301 CACAAAGCTG AAAAAGTTGT AAACATGATA TCAGGGACAA AATCTATAAC CCGCTATTAA TGGTGAAGAT ATTGACAGGG 10400
10401 CTGTATCTAT GATGTTGCAA ATCTCTAGGA CTTTAAGCAG TATTATCTAG AATTCCAATC AAATCTAATG GTAGGCTGAT 10500
10501 ATGTTGTCAA ATCTCTAGGA GTTCTCATTT GAAAGGGATA ATTATAGAAA AGTAACATCT CCTAGCATCA CTACATGTAT GGATGTCATA 10600
10601 TATGCAACTA GTTCTCATTT GAAAGGGATA TTTATACCTG AGTTCAGCAC ACAAGAGGTC AAAAAGCCCT TGGGTAGGGT 10700
10701 CGAGTACTCA AGAGAAAAAT ACACCAGAGT TTAGTACCTG TTTATACACAG ATCTGTCTTG GCTAGAAGCA TGAGATGGGT 10800
10801 GTATAAAGGG ACACCAGCAT ATTTCTTACA TAGATTATCT ACTCAACAAG AATGTGTAA AAATGTGTAA AACCTTTATT ACCTAGGTTT 10900
10901 ATGAGTCCAT TAATTGGTCT TAGAGTCGAG ATTTGGGAA TCAGTTCCAG CATTAGTGTA AACAAATTAC CATTCGACA 11000
11001 CTAGTCCTAT TAATCAAGCA CTAAGTCGAA GATTTGGGAA ACAGTTAGTT TCCAAAATGC GGAATTAGCA TAATGAGTGT 11100
11101 AGTAGAACAA TTAACAGTAA GAAGCCCAAA ACAGTTAGTT TTAATACCCC AATTAGAGAG CAGTGTTTCA AGGGAAAATC 11200
11201 AATTATAAAT TAGTAGATAA GATAACTTCT GATCAACATA TTTTAGTCC AATAGCACATT CACTAGGGAA AATGCTACCAC AGGGAAATTC 11300
11301 AATGGTCAGAA AACAGATCAG TTCTTAAATA AGAGAAGAAA TTATTCCAT GATCATGCTT GCATTAGCAT GTCATTGGTG CCCACTATAA 11400
11401 TGGGATATTA ACAGATGCAG GCATAGAATA TAATATTTTC GTTTATACTT TTATTCTCA TATGGACTT CAAAATATTC 11500
11501 CTATGTGTCT TTAAAACTAA ACTTTTATGT AGAATGTTCA GATCATGTAA TCAATAATCA TTATGGACTT GTAAAATTCC TATCACTAGT 11600
11601 GGATTGACAA TACTTTTTGG AGAAAGTTCA GCAAAGTAAT GTTTATATGAT AAAGGATAAT GTTTATATGAT GTAAAATTCC TATCACTAGT 11700
11701 AGGCTACATA GGGTTTTAAGA ACTGGTTTAT AGAGACAGTTG AATTGCAATGA AATACCTTGG CCGAAGGTGA TTTGGTTGAG 11800
```

Fig. 37C

HMPV strain 83 (continued)

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11801 ATCAAGTCAA TTAAAATCTA TTTGCAACTG ATAGAACAAA GCTTATTTTT AAGAATAACT GTTTTGAACT ATACAGATAT GGCACATGCT CTCACACGAT 11900
11901 TAATCAGAAA GAAGTTAATG TGTGATAATG CACTGTTAAC CCCAATTCA  TCCCCAATGG TTAACTTAAC TCAAGTTATT GATCCCACAA CACAATTAGA 12000
12001 TTACTTCCCC AAGATAAACAT TCGAAAGGCT AAAAAATTAT GACACAAGTT CAAATTATGC TAAAGGGAAG CTAACAAGAA ATTACATGAT ACTATTGCCA 12100
12101 TGGCAGCATG TTAATAGATA TAACTTTGTC TTTTAGTTCTA CTGGATGTAA AGTTAGTCTG AAAACATGTA TTGGAAAACT TATGAAAGAC CTAAATCCTA 12200
12201 AAGTTTTGTA CTTTATTGGA GAAGGAGCAG GAAATTGGAT GGCCAGAACA GCATGTGAAT ATCCTGATAT TAAATTTGTA TATAGAAGTC TGAAAGATGA 12300
12301 CCTTGATCAT CATTATCCTC TGGAATACCA GAGAGTGATA GGTGAATTAA GCAGAATCAT AGATAGTGGT CAATGGAAAC AACAGACGCA 12400
12401 ACTCAAAAAA CTCATTGGGA TTTGATACAC AGGGTAAGCA AAGATGCTTT ATTAATAACT TTATGTGATG CAGAATTTAA GGACAGAGAT GATTTTTTTA 12500
12501 AGATGGTAAT TCTATGGAGA AAACATGTAT TATCATGCAG AATTTGCACT ACTTATGGGA CGGACCTCTA TTTATTCGCA AAGTATCATG CTAAAGACTG 12600
12601 CAATGTAAAA TTACCTTTTT TTGTGAGATC AGTTGCTACT TTCATTATGC AGGGTAGTAA GCTGTCAGGT TCAGAATGCT ACATACTCTT AACACTAGGC 12700
12701 CACCACAACA GTTTACCTTG CCATGGAGAA ATACAAAATT CTAAGATGAA AATAGCAGTG TGTAATGATT TTTATGCTGC AAAAAAACTC GACAATAAAT 12800
12801 CAATTGAAGC TAATTGTAAA TCACTTTTGT CAGGGCTAAG AATACCTATA AATAAGAAGG AACTAGATAG ACAGAGAAGA TTATTAACAC TACAAAGCAA 12900
12901 TCATTCTTCT GTAGCAACAG TTGGCGGTAG CAAGATCATA GAGTCTAAGT GGTTAACAAA CAAAGCAAGT ACAATAATTG ATTGGTTAGA ACATATTTTA 13000
13001 AATTCTCCAA AGGGCGAATT AAATTATGAT TTTTTTGAAG CATTGGAGAA CACTTACCCT AATATGATTA AACTAATAGA TAACTTAGGG AATGCAGAGA 13100
13101 TTAAAAAACT GATCAAAGTA ACAGGATACA TGCTTGTAAG TAAAAAATGA AAATGATGA  ATAGATGACA AACTTCATACT ATTCTAAATT 13200
13201 AATTATTTGA TTATGCAATT ATATGATAGT TAATTAAAAT TAAAAATTAA AAATCAAAAG TTAAAATTTA AAACCTATCA TTAAGTTTAT TAAAAATAAG 13300
13301 AAATTATAAT TGAATGTATA CGGTTTTTTT GCCGT                                                                     13335
       |          |          |          |          |          |          |          |          |          |
       10         20         30         40         50         60         70         80         90         100
```

Fig. 37D

```
rHMPV-GFP
                 10          20          30          40          50          60          70          80          90         100
                  |           |           |           |           |           |           |           |           |           |
   1  ACGCGAAAAA  AACGCGTATA  AATTAAGTTA  CAAAAAAACA  TGGACAAGT   AGGAGCTGTT  CACCGGGGGTG  GTGCCCATCC                            100
 101  TGGTCGAGCT  GACGGCGGAC  AAGTCGCCCG  TGCCCTGGCC  CCTACGGCGT  CTACGGCGAG  AGCGGCCTGA  CTGACCCTGA  AGTTCATCTG                 200
 201  CACCACGGC   AAGTCGCCCG  TGCCCTGGCC  ACCCTCGTG   GAGAGTGCCGT  AGCCGCTACC  AGCGCCACAT  CCGACCACAT  GAAGCAGCAC                 300
 301  GACTTCTTCA  AGTCCGCCAT  GCCCGAAGGC  TACGTCCAGG  CGACTTCAAG  GACGACGGCA  ACTACAAGAC  CCGCGCCGAG  GTGAAGTTCG                 400
 401  AGGGCGACAC  CCTGGTGAAC  CGCATCGAGC  TGAAGGGCAT  CGACTTCAAG  GAGGACGGCA  ACATCCTGGG  GCACAAGCTG  ACAACAGCCA                 500
 501  CAACGTCTAT  ATCATGGCCG  ACAAGCAGAA  GAACGGCATC  AAGGTGAACT  TCAAGATCCG  CCACAACATC  GAGGACGGCA  GCGTGCAGCT                 600
 601  TACCAGCAGA  ACACCCCCAT  CGGCGACGGC  CCCGTGCTGC  TGCCCGACAA  CCACTACCTG  AGCACCCAGT  CCGCCCTGAG  CAAAGACCCC                 700
 701  GCGATCACAT  GGTCCTGCTG  GAGTTCGTGA  CCGCCGCCGG  GATCACTCTC  GGCATGGACG  AGCTGTACAA  GTAAGTTAAT  GGCGAAGAGC                 800
 801  AAATGTCTCT  TCAAGGGATT  CACCTGAGTG  ATCTATCATA  CAAGCATGCT  ATATTAAAAG  TACAATAAAG  AGAGATGTAG  GGACAAGTGA                 900
 901  AGCAGTGACA  CCCTCATCAT  TGCAACAGA   AATAACACTA  TTGTGTGGAG  TGCTAAGCAT  GCTGATTACA  AATATGCTGC  GCAACAAC                   1000
1001  ATACAATATA  TTAGCACAGC  TCTAGGATCA  GAGAGAGTAC  AGCAGATTCT  AAGAAACTCA  GGCAGTGAAG  TCCAAGTGGT  AGAAATAGGA                1100
1101  TGGGGAAAGT  TAAAAACAAC  AAAGGAGAAG  ATTTACACATA  AGCCAGATTCT  CACGGAGTAG  AGAAAAGCTG  GGTGGAAGAG  ACGTACTCCT                1200
1201  AACAATGGCA  ACTTTGCTTA  AAGAATCATC  AGGCAATATT  CCACAAAATC  AGAGGCCTTC  ACACCTATAA  TCTTATTATG  AAGCAAGAAA                1300
1301  TTAATATTTA  CCAAACTAGC  ATCAACTATA  GAAGTGGGAT  TAGAGACCAC  AGTCAGCGTG  AGCACCCGTG  TGCACTCAAA  TGTAGGTGCC                1400
1401  GGATGGACAT  ATGTCATTCT  GCTAGATCTT  TCTATGATTT  ATTTGAACAA  AAAGTGTATT  ACAGAAGTTT  GTTCATTGAG  AGATACCCTA                1500
1501  ATCCTCTACA  GGCAGCAAAG  CAGAAAGTTT  ATTGTTTAAT  ATATTCATGC  AGCTTACGG   TGCTGGTCAA  TATGGCAAAG  CATTAGGCTC                1600
1601  TCATCTAACA  ATATAATGTT  AGGCATCACA  TCTGAGTTAA  CTGTTCCAAG  GAAGTCATG   ACCTGGTGCG  AGAAAATGGGC  CCTGAATCTG                1700
1701  GGCTCCTACA  TTTAAGGCAA  AGCCCAAAAG  CTGGACTGTT  ATCACTAGCC  ACTTTGCAAG  TGTTGTTCTC  GGCAATGCCT  CAGGCTTAGG                1800
1801  CATAATAGGT  ATGTATCGCA  GGAGAGTGCC  AAACACAGAA  CTATTTTCAG  CTATGCCAAG  AGTTTGGAAG  AAAGCAATAA  AATTAACTTT                1900
1901  TCTTCATTAG  GACTCACAGA  TGAGAAAAA   AGGCTGCGCAG  AACACTTTCT  GACGACAGTC  AAAAATGATTA  TGCACTCAAA  AAAAAGTGGG                2000
2001  ACAAGTCAAA  ATGTCATTCT  CTAGAATCTT  TTCATGGGTA  AAATGTGAGT  AAAAATTGGCA  GAAGCTTTCC  AAAAAAATCATT  AAGAAAACCT              2100
2101  AGTCATAAAA  GATCTCAATC  TATTATAGGA  GAAAAAGTGA  ATGGAAGCGGC  TGAAACATTG  GAATTACCTA  CTATCAGTAG  CCGACCATAT                2200
2201  TGTCAGAGCC  GAAGTTAGCA  TGGACAGACA  AAGGTGGGGC  AAACAATCAA  AAACAATCAA  GAATTACCTA  AGTTATGGAT  AAGAAGAGTT                2300
2301  TACTGAGAAA  AGGGTGCTGC  CCTCCAGTGA  TGGGAAAACT  CCTGCAGAAA  ACCATCAACC  ATGCAAAGC   AGTTATGGAT  ATTTACACCA                2400
2401  AATGAACCAG  GAAAATACAC  AAAGTTGGAG  AGCATTGCTC  TAGAGTTGCT  GAAGAAGAAG  ATGCAGAATC  CTCAATCTTA  ACCTTCGAAG                2500
2501  AAAGAGATAC  TTCATCATTA  AGCATTGGAG  CCAGACTAGA  ATCGATTGAG  GAGAAAATTAA  GCATGAATTC  AGAACACTCA  ACATTGCTAC                2600
2601  AGCAGGACCC  ACAGCAGCAA  GAGATGGGAT  CGGACATGCA  ATCGATTGGCA  ACTAATAGCA  AGGCTAATAA  AGAAGAGCAA  GGGAAAAGCA                2700
2701  GCAGAAATGA  AATGAACCAG  CGGACAAAAA  TAGGAACAGG  CCGACAAAGG  TTAACTGAAA  GACAATAATAA  GCTCAACAAA  ATTGTTGAAG                2800
2801  ACGAGGTGAA  AAGTGGGTGAA  TCCGGACAGG  AAGAAGAACT  AAAAAGACACA  AGGCAAAGGA  TTAACTTAC   TGACATTTAC  CAGTTAATTA                2900
2901  TAAAAATAAA  AAATGGGACA  AGTGAAAAATG  GAGTCCTATC  ATCCCTTACA  ATCCCATTGT  CAGCAGCTGT  TCAAGTTGAT  TGTAGTTTAA                3000
3001  AGGACCTGTT  ACCTGCAAGC  CTAACAATAT  GGTTCCCCCT  AATACACACA  CAGCAGTTCT  GCTTGATCAG  CTAAAGACTC  CTAGTATAAC                3100
3101  TACTCTGTAT  GCTGCATCAC  AAAGTGGTCC  AATACTAAAA  GTTTCAGGCAT  TGCAGCAATG  TCTGTACTTC  CCAAAAAGTT  TGACTATAAC                3200
3201  GCGACTGTAG  CACTGACGA   ATATAGCAAA  TTAGAATTTG  ACAAACTTAC  GGCCCAGGG   GTAAAAACAG  TTTACTTAAC  CCATATGGAT                3300
3301  TGGTATCAAA  GTTGTTGTGAG  TCGGCCAAAC  CAGTTTGGCAA  CATTATGCGA  AGTCTAATCG  TTTTATGGAT  CTAGAAAAGA  ACACACCAGT                3400
3401  TACAATACCA  GCATTATCA   AATCAGTTTC  TATCAAGGAG  AGCTGCAATA  AGCAGTGAAG  TTTATGGAT   AGCAGTGAAG  TCTAACACAA                3500
3501  GCCAAAATTG  CACCTTATGC  GGGACTGATC  ATGATTATGA  TCCCAAAGGC  ATATTGCAATA  AAGGAACAAG  AGCTTGGAGC  GTTATAGTAG                3600
3601  AACTAGGAGC  ATATGTCCAG  GCTGAAAGCA  TAAGTAAAAAT  TCCCAAAAGGC  AAGGACCCAA  TGGGACCCAA  ATATGTGCTG  AACAGCCAAG                3700
3701  CAACCTGACC  AAGAACTACC  AACTCTATTC  TATAGACTAA  TTTTAGTTAT  ATAAAAATCA  AGAATGCTAG  AGTTAGAATA  CAATCAAGAA                3800
```

Fig. 38A

rHMPV-GFP (continued)

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3801 CGGGACAAAT AAAAATGTCT TGGAAAGTGG TGATCATTTT TTCATTGCTA ATAACACCTC TAAAGAGAGC TACCTAGAGAG AATCATGTAG 3900
3901 CACTATAACT GAGGGATATC TTAGTGTTCT GAGGACAGGT TGGTATACCA ACGTTTTTAC ATTAGAGAGTG GGTGATGTAG AAAACCTTAC ATGTTCTGAT 4000
4001 GGACCTAGCC TAATAAAAAC AGAATTAGAT CTGACCAAAA GTGCACTAAG AGAGCTCAAA ACAGTCTCTG CTGACCAATT GAACAAATTG 4100
4101 AGAATCCCAG ACAATCTAGG TTTGTTCTAG GAGCAATAGC ACTCGGTGTT GCAACAGCAG AGCAGGTGTT GCAATTGCCA AAACCATCCG 4200
4201 GCTTGAGAGT GAAGTCACAG CAATTAAGAA TGCCCTCAAA AAGCAGTATC TACAGTGTTC GAGTGTTTGGC GAGTGTTGGC AACTGCAGTG 4300
4301 AGAGAGCTGA AAGACTTTGT GTTGTGCGGC AGCAAGGTGG GACAATCTTT GACTAAAAAT ACTGTATATC GGCCGTTAGC AACTAGCCAG 4400
4401 TCAACAGAAG GTTTCTAAAT TTAACTCGTG CAATCAACAA AATAGACACC AATAGACAAC ACTGTATATC AGCTAAGCAA TTCAGTCAAT 4500
4501 GGCCGTTTCT AACATGCCGA ACAAATAAAA TTGATGGCGG AGAACCGTGC GAAGAAGGGGT ACAGATGCTG ACAGATGTCT AACTAGCCAG 4600
4601 TACGGGAGCT CCGTAATTTA CATGTGCAGG CTGCCAATCT TTTGGCGTTAT TAAAAGGGGT CCCTTCTTGT GATAGGGGTC 4700
4701 AGGGAAAACTA TGCTTGCCTC TTAAGAGAAG ACCAAGGGTG GGGAATTAAT CAACTGTTTA TAAAAGCAGC CCCTCCTAAT TCCGGAAAAA 4800
4801 AGGAGACCAT GTCTTTTGCG ACACAGACAG TATCAGTATG GGGAATTAAT AATCAACATC AACATATCCA GAGAAAGACT GTGAAACAAG 4900
4901 GTCAGCACAG GAAGACATCC GAAGACATCG TTTGCACTGT GTTGCTGTT AAGGAGTAAG CTGTTCCATT GGCAGCAACA CCCATGCAAA 5000
5001 GAGTAGGGAT TATCAGTATG CTGAACAAGG GTTGCTCCTA CAAGATGCAG ACACAGTGAC CTGTTCCATT GGCAGCAACA ACTGCAGTG 5100
5101 AGTTGAGGGT CATCAAGCAG CTGAACAAGG GTTGCTCCTA AATAGACAAC ACTGTATATC ACTGTATATC AGCTAAGCAA 5200
5201 GTTTTTGAGA ACATTGAAAA CAGCCAGCCC TTGGTAGATC AATCAAACAG AGTGCAGAGA AAGGGAATAC TCAATGTTGC ACTTGACCAA 5300
5301 TTCTAATTGC TGTCCCTTGGC TCTAGCATGA TCCTAGTGAG CATCTTCATT ATAATCCTAAGC TGGCTTCATC ATTGTAATAA 5400
5401 TGTCACAAAC AATGGCTTCA TACCACACAG TTAGTTAATT AAAAATAAAA ACCAAAGAA GCACCTCCAG AGCTGAGTGG 5500
5501 AGTGCGGGGC AAATGCAACA GAGGAAGTGA GTGTAAGTTT AACCACAATT AACTTATTAA AAGGCTCCAT AAGGCTCCAT GCAAATATGA 5600
5601 AATCAGCTTT TAAGGAACAC TGATAGAGCT GATGGCCTAT CAATAATATC TACTTATTAA TAAGATCAAA CTATCTATTA CTATCTATTA 5700
5701 ATGTGGTTCA AGGTTATATT GATGATGAAC AAAAGCATAA GTCTCACACA GAACGCAAGA TTTTGTTCTA GGTTCCACCA 5800
5801 TAGGCAGGCT AGAGATAGCA AGGTTATATT CTTATCTTAC ATAACTTAAT ATGGAGATGA GCAAAAACTCC AGCATCTTTA 5900
5901 ATCAACAATC TCAAAAAGACT GCCGAGAGAA AAACTGAAAA GCTGATAATT GACTTATCAG CAGGGCGCTGA CAATGACTCT TCATATGCCC 6000
6001 TGCAAGACAG TGAAAGCATT AATCAAGCAT AGTGGCATG CATTACTATA GAGGTTGATG AAATGATATG GAATTAAAAG 6100
6101 AAGCTTTGTC CGATGGGGATA GTGAAGTCTC ACACCAACAT GATTAATATT TATTTAGAAA ACATATGTC AAGGCTTACT TAAGTTAGTA 6200
6201 AAAACACATC GTAAAGCATT AATCAAGCAT GATAACATTA CATTACTATA ACATGTACTC ACTGTACTC AATAATCAAA 6300
6301 GACCATTCTG GTAAAGTGCT TATTGCACTT AAGTTAATAT TAGCTTTACT AACATTTTTC ACAATAACAA TCACTATAAA GTAGAAAACA 6400
6401 ATCTACAAAT ATGCCAGTCA AAAACTGAAT CAGACAAAGA AGACTCACCA AACATTTTTC AACCAAGACC ACTCTAGACC ATGATATAAC 6500
6501 ACAGTATTTT AAAAGATTAA TTCAAAGGTA TACGGATTCT GTAATAAACA AGCAGAAATC CTGAAAAATA AGCAGAAATC TATAACAACA 6600
6601 TATAAATTTT TATGCTTTAA ACCTGAGGAC GTAATAAACA TCAAAAATCA GATCTATGCA AAAATCAGCA AAAATCAGCA GCTGAAGCAT 6700
6701 ATCATACAGT AGAATGCCAT TGCATATACA TGCATATACA CAATTGAGTG GAAGTGCTAT CCAATCTTGA ATGTTAAAAC TAGACTAGGA 6800
6801 TCCGTCTAAG ACTATCAGTT CAATAGTTTA GTTTATTTAA AATATTTGAG CACCACTCGA TAGATTGGCG ACTTCATAGC AATTAACAGC 6900
6901 TTAATTATAA TTAAAACATT ATTTAAAAATC GTAACTATTT AATTTACAAA CCAATCTTGA ACTTCATAGC AATTAACAGC 7000
7001 ATTCGAGCAA TAGACATGCT CAAAGCAATC GTGAAAAATC AATTACAAA AAATATGGGA CAAGTAGTTA AGTAGAGAAC AGTAGAGAAC 7100
7101 TGAGTATAGC TCTCAATATC TATCTGATCA TAAACTATAG CTTCTTTAAT CTTCTTTAAT CCTCATAGGA ATAACTACAC 7200
7201 CAAGGAAGCT TCAACAATCT CCACACCAGC AACATCCAAC CCCAAATGC AATCAGAACA CCACCACCA CAGAATCCAA CAGAATCCAA 7300
7301 GCATCAGTGA GCCCATCAGA AACAGAACCA TCCTCCGTAG ACACACACT ACACACACT CCCACACACT CAACCCCGCA 7400
7401 GAACAAAGAC AAAACCGACA GTCCACACAA GCACAGGAAA GAAACAAATC AAAATCAGCA AGCAAACCAGC AGTGAAAGCA 7500
7501 CACCACTTTC CGCATGAGCA AATCCCCACC ACGGGCACCC ACGGAGGCAA ACGGAGGCAA ACGGGCACCC TCCGCAGAGC 7600
7601 GCGAACCCAC AGGCATCTGT AAGCACATCA AGCACAATG ACAACCAAAA ACAACCAAAA ATCATGAAGA AACAGGTTCA 7700
7701 CATGTAGGTA CCAACAATCA AGAAAACCAA CGAACAGCAA AAAACAGCAA TATAAAAACA AATTAGTTAA GAGATAGCTC TAAAGTAAAA 7800
```

Fig. 38B

rHMPV-GFP (continued)

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7801  CTTTTGCCCA CATACTAACA ACATCACAAC CATCCTCAAGA AAAGAAACTG  GGCAAAACAG CATCCAAGAG ACAAATAGCA ATGGATCCTC TTAATGAATC  7900
7901  CACTGTTAAT GTCTATCTCC CTGATTCGTA CCTTAAAGGA GTAATTTCTT  TTAGTGAAAC TAATGCAATT GGTTCATGTC TCTTAAAAAG ACCCTACTTA  8000
8001  AAAAATGACA ACACTGCAAA AGTTGCCATA GAGAATCCTG TTATTGAGCA  TGTGAGACTC AAAAATGCAG TCAATTCTAA AATGAAAATA TCAGATTACA  8100
8101  AGGTAGTAGA GCCAGTAAAC ATGCAACATG AAATAATGAA GAATGTACAC  AGTTGTGAGC TCAATTCTAA GAAACAGTTT TTAACAAGGA GTAAAAACAT  8200
8201  TAGCACTCTC AAATTGAATA TGATATGTGA TTGGCTGCAA TTAAAGTCTA  CATCAGATGA TACCTCAATC CTAAGTTTCA TAGATGTAGA ATTTATACCT  8300
8301  AGTTGGGTAA GCAACTGGTT TAGTAATTGG TACAATCTCA ATAAGTTAAT  TTTGGAATTC AGAAGAGAGG AAGTAATAAG AACCGGTTCA ATCTTATGCA  8400
8401  GGTCATTGGG TAAATTAGTT GATGTGATGT CATCATATGG ATGTATCGTC  AAGAGCAACA AAAGCAAAAG AGTGAGCTTC TTCACATACA ATCAACTGTT  8500
8501  AACATGGAAA GATGTTAAAC TAATGAAACTG TTTTGTATAT GGGTAAGCAA  TAGTCTGAAT GAAAATCAGG AAGGGCTAGG GTTGAGAAGT  8600
8601  AATCTGCAAG GTATGTTAAC TAATAAACTA TATGAAACAT CTCAATTCAGT  GCTAAGCTTA TGTTTCAATG AAGGTTTCTC ACTTGTGAAA GAGTTCGAAG  8700
8701  GTTTTATTAT GAGTGAGATC CTTAGGATTA CTGAACATGC TCAATTCAGT  ACTAGATTTA GAAATACTTT GTTAACAGATC AATTAACAAA  8800
8801  ATTAAAAAAT AAAAACAGAC AATTATTAAT TCAGAGTTCA TTAGAAAAATA  ATGATTATCC AATGTATGAA GTTGTACTTA AATTATTAGG AGATACTTTG  8900
8901  AGATGTATCA AATTATTAAT CAATAAAAAC TTAGAGAAATG CTGCAGAATT  ATATTATATA TTCAGAATTT TTGGTCATCC AATGGTAGAT GAAAGAGATG  9000
9001  CAATGGATGC TGTCAAATTA AACAACAAAA TCACAAAAAT CCTAAGGTTG  GAGAGCTTGA CAGAACTAAG AGGGGCATTC ATATTAAGGA TTATCAAAGG  9100
9101  ATTTGTGGAC AACAACAAAA GGTGGCCCAA AATTAAAAAC TTAAAAAGTGC  TTAGCAAAAG TACTTCAAAG CCCCAGTCAA TTATCAAAG  9200
9201  CTCGAATTAA GTGAACAAGA CTTTCTAGAG CTTGCTGCAA TACAATTTGA  ACAAGAGTTT TCTGTTCCTG AAAAAACCAA TCTTGAGATG GTATTAAATG  9300
9301  ACAAAGCCAT ATCACCTCCT AAAAGATTAA TATGGTCTGT GTATCCAAAG  CTGAGACGAT AAAAAATCGA TATTTAGAAG AAACTTTCAA  9400
9401  TGCGAGTGAT AGTCTCAAAA CAAGAAGAGT ACTAGAGTAC TATTTAAAAG  TGATCAAAAG GAACTTAAAA GTTATGTAGT TAGACAAGAA  9500
9501  TATTTAAACG ATAAGGAGCA CATTGTCTCA TTAACTGGAA AAGAAAGAGA  ATTAAGTGTA GGTGAAATGT TTGCTATGCA ACCAGGAAAA CAGCGACAAA  9600
9601  TACAAATATT GGCAGAAAAA GAACTTTCTT ATAACATTGT ACCTTTCTTC  CCGGAAACCT TAACAAGTA TGGTCATCTA GATCTTCAGA GAATAATGGA  9700
9701  AATCAAATCA GAACTTTCTT CTATCAAAAC CAGAAGAAAT CAGAAGAAAT  ATAATTACAT TCCATAGTAA CAGATTTGAG CAAGTTCAAC  9800
9801  CAAGCCTTTA GATATGAAAC TACAGCGATC TGTGCGGATG TAGCAGACGA  ATTAATTACACT ATTCTGTTG GTTACATCTT ATCGTTCCTA  9900
9901  TGACTACAAT GATATGTGCC ATTGAAGGAT GGTGTCAAAA ACTCTGGACA  GAATAAGATA TAGATAAGAT AGAAGAGCAA AGTGGTCTAT ATAGATATCA 100000
10001 CATGGGCCGGT ATTGAAGGAT GGTGTCAAAA ACTCTGGACA ATGGAAGCTA  TATCTTTATT GGATGTTGTA TCTGTAAAGA CACGGTGTCA AATGACATCT 10100
10101 TTATTTAAACG GTGACAACCA ATCAATAGAT GTAAGTAAAC CAGTCAAGTT  ATCTGAAGGT TTAGATGAAG TGAAGGCAGA TTATCGCTTA GCAAGGTGAT 10200
10201 TGCTAAAAGA AATAAGAGAT GCATACAGAA ATATCCACCC TATAAAAAAG  GAAGGGGAAA CATATATATC AAGGGATCTT CAGTTTATAA GCAAGGTGAT 10300
10301 TCAATCTGAA GGAGTGATGC ATCCTACCCC TATATAAAAG GTCTTGAGAG  GATAAACACA ATATTAAGAA ACTTCTGGCT GTATAACTTA TAGTGCTGAG 10400
10401 TCAATAGGGA GTCTATGTCA AGAATTAGAA TTTAGGGGAG AAAGCATAAT  AGTTAGTCTG CATCAGTGCA GAGATTTTTT GAAATTAAAA GGGAAAATGA 10500
10501 AATCAAAGCA ACATCCTTTG GCAGGGAAAC AGTTATTCAA ACAACTAAAT  CAGTAGTCTT TTCTATAGAA TTCTCAAAGC GTTTTTTAACT ATAGAAAAAA 10600
10601 GGTAGTAGAT CTATGGATGA ACATACCAAT TATTCTGTTA GGAGGAGATC  CAGTAGTCTT TTCTATAGAA TTCTCAAAGC GGACCCCTGA TTTTTTAACT 10700
10701 GAGGCAATCA GCCAATGTAGA TACACTGACA AAAATATCAG CTAACATAAA  AAATGAAAACG AACAAGTAGT CTTCAAAGAGC CTTACTATCA ATAGAAAAAA 10800
10801 ATGAAACGTGC TACACTGACA AGTCTTTCCC CAAATAATGA TTTCAGTGAT  ACTACAGCAG AAATGAACGAA TCATTGCAGA AAACATAACA CAGCAGTTAC 10900
10901 CAGTATCTTA CTCTTTTATC GTGACAACCA ATCAATAGAT GTAAGTAAAC  ACTACAGCAG AAATGAACGAA TCATTGCAGA AAACATAACA 11000
11001 CCTGTTTATC GAGAACATCC GCTATTAATG GTGAAGATAT TGACAGGGCT  CAAAGCTGAA AAAGTTGTAA ACATGATATC AGGGACAAAA TCTATAACCA 11100
11101 ACTTATTACA GAGAACATCC AGTATAGAAA TTCCAATCAA ATCTAATGGT  GTATCTATGA TGTTGGAGAA CTTCTATTA TATTGTCAGT 11200
11201 AGTTGTTGAT AGTATAGAAA TAACATCTCC TAGCATCACT ACATGTATGG  GTTGTCAAAT CTCTAGGACT TTAAGGAGA TAATATGGAA TTCAGCACTG 11300
11301 ATAGTTGGAG TAACATCTCC TAGCATCACT ACATGTATGG AGTGTCATAT  TGCAACTAGT ATCTCATTGA AGGGGATAAT CATCATGAAA TTCAGCACTG 11400
11401 ACAGAACTAC AAGGGGTCAA AGAGCAGCAG TAGAACGCAT GAAGCAAAAT  AGTAAGGGAC CTTATTAC TAAGGCTTG TCAACAAGAT CTGTCTTGGG 11500
11501 AAAAACAACAA AGAGAACAGA TTAGCTACAA ATGTGTAAAA TCTTTATTAC  ATAAAGGGAC CACCAGGCTTG CGACGATTAC GATTATCTGT CAGTAGTAGA 11600
11601 AGTTTAGGCA TTGCTACACAA ATGTGTAAAA CCTTTATTAC CTAGGTTTAT  GAGTGTAAAT TTCTTACATA AGTCCTATTA 11700
11701 TCCCAGTCATC AGTTCCAGCT TATAGAACCAA CAAATTACCA TTTCGCACACT  AGTCCTATTA ATCAAGCACT AAGTGAGAGA TTTGGGAATG AAGATATTAA 11800
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Fig. 38C

rHMPV-GFP (continued)

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11801 CTTGGTCTTC CAAAATGCAA TCAGCTGTGG AATTAGCATA ATGAGTGTAG TAGAACAATT AACAGGTAGA AGCCCAAAAC AGTTAGTTTT AATACCCCAA 11900
11901 TTAGAAGAAA TAGACATTAT GCCACCACCA GTGTTTCAAG GGAAATTCAA TTATAAATTA GTAGATAAGA TAACTTCTGA TCAACATATC TTTAGTCCGG 12000
12001 ACAAAATAGA TATGTTAACA CTAGGGAAAA TGCTCATGCC CACTATAAAA GGTCAGAAAA CAGATCAGTT CTTAAATAAG AGAGAAAATT ATTTCCATGG 12100
12101 AAACAATCTT ATTGAGTCTT TATCAGCAGC ATTAGCATGT CATTGGTGTG GGATATTAAC ATAGAAAATA ATATTTTCAA TTGGGGATCT GAAAGACTGG 12200
12201 GGTGACGGGT TTATATCAGA TCATGCTTTT ATGGACTTCA AAATATTCCT TTTTATGTAG AATGTTCAGC AAAGTTATGT TGGTTTATAG CAAGGGAAAA 12300
12301 ACATTAAAGA TGAAGATATA GTAGATGAAT CAATAGATAA AAAACTAAAC CTTTTTGGAG AATGTTCAGC AGCAGTTGAG ATCAGCTGAT TTGAACCAAA 12400
12401 GGTTAAGAAA AGGATAATGT TATATGATGT AATAATCCTA TCACTAGTAG AAAATCTATT TGCAACTGAT AGCAGTTGAG AGAACAAAGC ATCAGCTGAA 12500
12501 TTGCATGAAA TACCTTGGAT TGTCAATGCC GAAGGTGATT CACACGATTA AGTTAATGTG TGATAATGCA AGTTAACCCC TGGTTTATAG TTATTTTTAA 12600
12601 GAATAACTGT TTTGAACTAT ACAGATATTG CACACGATTA CAATTAGATT GTTAATGTGT CATTGGGATT TGATAATGCA AGTTAACCCC CAATTTCATC 12700
12701 CCCAATGGTT AACTTAACTC AAGTTATTGA TCCCACAACA AGTTAATGTG GATAACATTC ACTTTGTCTT TAGTTCTACT GGATGTAAAG CACAAGTTCA 12800
12801 AATTATGCTA AAGGGAAGCT AACAAGAAAT TACATGATAC TATTGCCATG AATAGATATA ACTTTGTCTT AGGAGCAGGA AATTGGATGG CCAGAACAGC 12900
12901 TTAGTCTGAA AACATGTATT GGAAAACTTA TAGAAGTCTG AAATCCTAAA TTATTGGAGA TTATCCTCTG GAATACCAGA GAGTGATAGG TGAATTAAGC 13000
13001 ATGTGAATAT CCTGATATTA AATTTGTATA TAGCATTTCA TAGGAAGACC AAAGATGACC CAGACGCAAC GAATACCAGA GGTAAGCAAA GATGCTTTAT 13100
13101 AGAATCATAG ATAGTGGTGA AGGAAAACAA ATGGAAACAA ATGGGATGA CAGAGGATGA CATTGGGATT TGATACACAG GGTAAGCAAA GATGCTTTAT 13200
13201 TAATAACTTT ATGTGATGCA CAGAGGATGA TTTTTTTAAG CATTGGGATT TATGGAGAAA ACATGTATTA TCATGCAGAA TTTGCACTAC TTTGCACTAC 13300
13301 TTATGGGACG GACCTCTATT TATTCGCAAA GTATCATGCT AAAGACTGCA ACCTTTTTTT GTGAGATCAG TTGCTACTTT CATTATGCTT CATTATGCAG 13400
13401 GGTAGTAAGC TGTCAGGTTC AGAATGCTAC TATGCTGCAA AGAATGCTAC TTACCTTGCC ATGGAGAAAT ACTTTGTCA GGGCTAAGAA AAGATGAAAA 13500
13501 TAGCAGTGTG TAATGATTTT TATGCTGCAA ATTAACACTA CAAAGCAATC ATTGTAAATC ATTTTGTCA GGGCTAGCA AGATCATAGA TACCTATAAA 13600
13601 TAAGAAGGAA CTAGATAGAC AGAGAAGATT ATTAACACTA CAATAAATCA AATTAACTGA ATTGTAAATC GGCGGTAGCA AGATCATAGA GTCTAAGTGG 13700
13701 TTAACAAACA AAGCAAGTAC AATAATTGAT TGGTTAGAAC CAAAGCAATC TTCTCCAAAG GGCGGTAGCA AGATCATAGA TTTTGAAGCA TTGGAGAACA 13800
13801 CTTACCCTAA TATGATAGAT ACTAATAGAT ACTTAGGGAA TTCATACTAT TCTCCAAAGG GGCGGTAGCA ATTATGATTT TTTTGAAGCA AAAAATGAAA 13900
13901 AATGATGAAG ATGACAAAAT AGAGACAAAT TTCATACTAT ATGCAATTAT TCAAAGTAAC AGGATACATG ATGATAGTTA CTTGTAAGTA AAAATTAAAA 14000
14001 ATCAAAAGTT AAAATTTAAA ACCTATCATT AAGTTTATTA ATTATAATTG AATGTATACG GTTTTTTTGC CGT 14083
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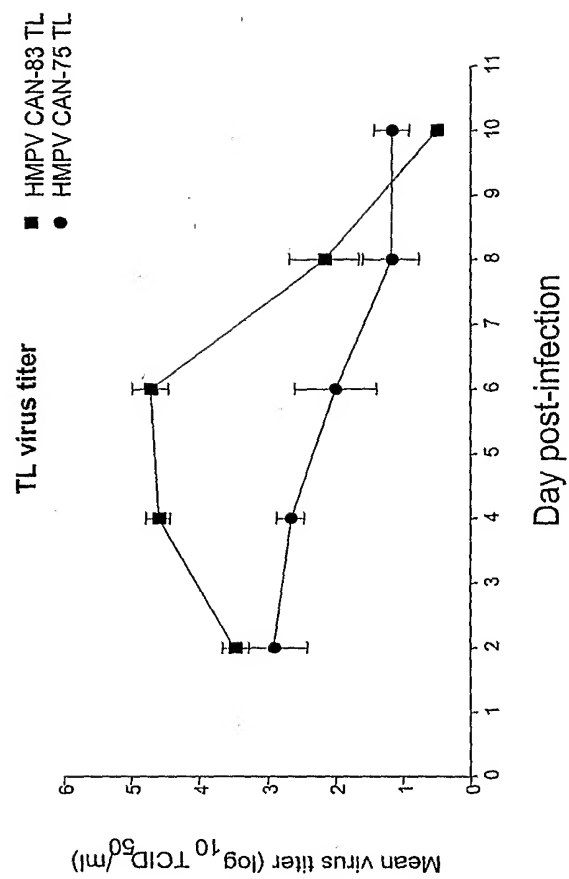
Fig. 38D

HMPV strain 75

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           10         20         30         40         50         60         70         80         90        100
     |          |          |          |          |          |          |          |          |          |
   1 ACGCGAAAAA AACGCGTATA AATTAAATTC CAAACAAAAC GGGACAAATA AAAATGTCTC TTCAAGGGAT TCACCTAAGT GATCTGTCAT ATAAACATGC  100
 101 TATATTAAAA GAGTCTCAAT ACACAATAAA AAATATGCTG TTCTAACCAA CTGCAGTGAC ACCTTCATCA TTGCAGCAAG AGATAACACT TTTGTGTGGA  200
 201 GAGATTCTTT ACACTAAACA TACTGATTAC CAGAGATAGG GACATACTCT GATACAATAT ATTTGCACGG CTCTAGGATC AGAAAGAGTA CAACAGATTT  300
 301 TAAGAAATTC AGGCAGTGAA GTTCAGGTGG TTCTAACCAA AAATAGACAA GTAAAGATGG TAAAGGGGAA AAGGAATCAT TCAGGTAACT GTTTAGATAT  400
 401 ACATGGAGTG GAAAAGAGTT GGGTAGAAGA AATGAGACAA GACTTTGCTA ACAATAGGAA AAGGAATCAT CAGGAATCAT TATATATAAT CCCACAAAAC  500
 501 CAGTTAGAAG CAGCACCAGA CACACCAATA ATTTTATTAT GTGTAGGTGC AGGGTAGAGC CAAAATTGGC ATGGGCAGAC ATGGGAAGTA CTAGAGACTA  600
 601 CAGTTAGAAG AGCTAACAGA GTGCTAAGTG ATCGGCTCAA ACTAAACTAG TACCGAAGAT TGCTAGATCT TTCTATGAAC TGTTTGTAAA TATTTGAGCA  700
 701 GAAAGTGTAT TACAGGAGTC TATTCATTGA AAGATACCCT AGGTAGATA AGGAAGCAAA GCAGAAAGTT TGTTTGTAAA TATATTTATG  800
 801 CAAGCTTATG GAGCCGGTCA AACAAATGCTA AGGTGGGGTG AGAAAATGGG ATCATCTAAC AACATAATGC TAGGACATGT GTCTGTGCAA GCTGAATTGA  900
 901 AGCAAGTTAC AGAGGTTTAT GATTTGGTGA GAGAAAATCT TCCTGAATCT GGGCTTTTAC ATCTAAGACA AAGTCCAAAG GCAGGACTGT TATCGTTGGC 1000
1001 CAATTGCCCC GATTTTGCTA GTGTTGTTCT TGGTAATGCT TCAGGTCTAG GTATAATCGG AATGTACAGA GGAAGAGTGC CAAACACAGA GCTATTTTCT 1100
1101 GCAGCAGAAA GTTATGCCAG AAGCTTAAAA GAAAGCAACA AAATCAACTT CTCCTCATTA GGGCTCACAG ACGAAGAAAA AGAAGCTGCA GAACACTCT 1200
1201 TAAACATGAG TGATGACAAT CAAGATGATT ATGAGTAATT AAAAAAACTGG GACAAGTCAA AATGTCATTC CCTGAAGGAA AAGATATCCT GTTCATGGGT 1300
1301 AATGAAGCAG CAAAAAGCTTTC CAGAAATCAC CAGAAATGAC ATCATCTACA AGGTCACAAA AGAACCCAGT CTATTGTAGG GGAAAAAGTA AACACTATAT 1400
1401 CAGAAAACTCT AGAGCTACCT ACCATCAGCA AACCTGCACG ATCCATCTACA CTGCTAGAGC CAAAATTGGC ATGGGCAGAC AGCAGCAGAG CCACCAAAAC 1500
1501 CACAGAAAAA CAAACAACCA AAACAACAGA TCCTGTTGAA GAAGAGGAAC TCAATGAAAG ATGGGAAGAC ATGGGAAGTA TCCCGCAGAG 1600
1601 AAAAAAATCAA AATCTCCAAC CAATGTAAAA AAGAAAGTTT CCTTCACATC GGGAAATATA CCAAACTAGA AAAAAGATGCC CTAGATTGC 1700
1701 TCTCGACTAA TGAGGAAGAA GACGCAGAGT CCTCAATCTT AACCTTTGAA GAGAGAGACA AAGCATTCAC TCAGAGATGG AATGATTGGT AATCAATAGA 1800
1801 AGAGAAGCTA AGCATGATAT TAGGACTGCT TCGTACACTT AACATTGCAA ATGGAATGGAA GCTAGATAGG TCAGAGATGC AATGATTGGT ATAGGTAATG 1900
1901 ATAAGAGAAG AACTAATAGC AGAAATAATA AAAGAAGCAA ATGAATCA AAGGTCAAAA GAAGAAGAAC CAAAAGAAAC AAAAGAAAAC 2000
2001 GCAGTGTAAA ACTAACCGAG AAGGCAAAAG AACTTAATAA CCAGTTAATC AAGCGGTGA ATCAGAAGAA GGAGTCCTAT CTAGTGGACA CTAGTGGACA 2100
2101 TCAGGATAAC AATCAAGGAG AAGATATTTA CCGAGTTAATC ATGGGGCAAG AAGTCAAGAT AAGTCAAGAT TTTGACAATA TATTCCAAGC 2200
2201 CTTATCAAGG CATTCCCTAC ACAGCTGCTG TTCAAGTTGA GCTAAAGACC TACCAGCAAG AGTGCTGCAAG CAGAATGGTC CAATACTCAA GGTAAATGCA 2300
2301 CAACACACCA CCAGCCGGTTT GTCTCGTATC TGCTAAAGAA TTGACAATAA TGCTGCATCA CAACTGTAAA ACTTGACTTT GACAAGTTAA 2400
2401 TCAGCTCAGG CGGTTGCTAT GTCTTATTGA CCCAAAAAAT TCGAAGTAAA ATGGTGTCAA AATTTGTGAG TTCAGTTGCA ACAAGACACA 2500
2501 CGGTTTGCGA TGTTAAAACA ACTTCATGGA CAACCATGAA ACCATATGGG GCAACTATCG AGCATTCATA AAGTCAGTTT CAATCAAAGA GAGTGAGTCA 2600
2601 TGATCTAATT GCACTGTGTG ACTTCATGGA CCTAGAGAAA ANTTAACCTG CATTAACACA TGACAATACC AGGACTCAAT CATGATCATG ACCATGAACA 2700
2701 GCCACTGTTG AAGCTGCAAT AAGCAGTGAG GCCGACCAAG CATTAACACA AGTAGTAGTA CAGGACTAAT CAGCCGAGAGC TCTGCAAGAG 2800
2801 ATCCAAAAGG TATATTCAAG AAAACTAGGAG CTGGAACACA AGTGATAGTA TAAAAATAAC AGCCTAGGGGC CATATGTTCA ATCAGCAGAGC TCTGCAAGAG 2900
2901 CTGGAGTCAC CAAGGAACAA TGATATGTACT AAAAATCCAGA TGTCCTAATC AATAATTGCT TATATATTGCT TAAAGATCAA TGAGCTTATT 3000
3001 ATTATAGTTA TATAAAAAAA TTTAGAACTA GGAAGGTATT GGGACAAGTA AAAATGTCTT GGAAAGTCTT GATTATCATT TCGTTACTCA 3100
3101 TAACACCTCA GCACGGACTA TTAGAAGTT ATTTAGAGAA ATCATGTGAT GACCTAGCTT AAGGATATCT CAGTGTTTTA AGAACAGGTT GGTACACCAA 3200
3201 TGTCTTTACA TTATGCACTT GTGATGTTGA AAAATCTTACA GCGAGAGAAG AACCCCAGAA AATCAAAGGT TAACCAAAAG TGCTCTAAGA 3300
3301 GAACTCAAAA CAGTTCCTGC AGCAGTCACA AACAACCTAG ACCTAGGACTT CTTGAGAGTG TGCAATGACT TGTCAATAGT GCTCTCAAAA CAACCAATGA 3400
3401 CCACAGCAGC AGCAGTCACA CGAGCATTG GGAAGAAGT AAGTGAATGC GAGGGCTGAA AGTCAAAGAT AGCAAAAACC TGACTAGTGC AATTAACAAG 3500
3501 GGCAGTATCC ACACTAGGAA ATGGAGTGCG AGTCCTAGCC CAACAGAAGA TTCCTAAAATG TTGTGCGGCA GTTTTCAGAC AATGCAGGGA 3600
3601 AACAAATGTG ACATTGCTGA TCTGAAGAATG GCTGTCAGCT TCAGTCAATT TCCTAAAATG TTGTGCGGCA GTTTTCAGAC AATGCAGGGA 3700
3701 TAACACCAGC AATATCATTG GACCTAATGA CTGATGCTGA GCTGGCCAGA ATCTGCAGGA ACATGCCAAC ATCTGCAGGA AATGCAGGGA TAATGTTAGA 3800
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Fig. 39A

HMPV strain 75 (continued)

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3801 GAACCGTGCA ATGGTGAGGA GAAAAGGATT TGGAATCTTG ATAGGGGTCT   ACGGAGAGCTC CGTGATTTAC ATGGTCCAGC TGCCGATCTT TGGTGTCATA 3900
3901 GATACACCTT GTTGGATAAT CAAGGCAGCT CCCTCTTGTT CAGAAAAAGA   TGGAAACTAT GCTTGCCTCC TAAGAGAGGA TCAAGGGTGG TATTGTAAAA 4000
4001 ATGCAGGATC CACTGTTTAC TACCCAAATA AAAAAGACTG CGAAACAAGA   GGTGATCATG TTTTTTGTGA CACAGCTGCA GGGATCAATG TTGCTGAGCA 4100
4101 ATCAAGAGAA TGCAACATCA ACATATCCTA AACCAACTAC CCATGTGCAA   TCAGCACAGG AAGACACCCT ATCAGCATGG CTGCCTCATC ACCTCTCGGT 4200
4201 GCTTTGGTGG CTTGCTACAA AGGGGTTAGC TGTTCAATTG GCAGTAATCG   GGTTGGAATA ATCAAACAAC TACCTAAAGG CTGCTACTAC ATAACTAACC 4300
4301 AGGACGCAGA CACTGTAACA ATTGACACCA CTGTGTATCA ACTAAGCAAG   GTTGAGGGTG AACAGCATGT AATAAAAGGG AGACCAGTTT CAAGCAGTTT 4400
4401 CGATCCAATC AAGTTTCCTG AGGATCAGTT CTTGATCAAG GGCTTGTGCG   TCTTTGAAAG CATTGAAAAC AGTCAAGCAC CCAAATTCA  GTCAAACAAA 4500
4501 ATTCTGAACA GTGCAGAAAA AGGAAACACT GGCTTCATTA TTGTAATAAT   AAAGCACTAA ATGGGATAGC AAAATCACAC ACAGTTGTTA TTTAGAAAAT 4600
4601 TAATCAAAAA AACAAGGAAA CCCACAGGGG CACCTCCAGA GCTGAATGGT   AATGGGATGA AAAGACAATGA ATGACAATGA AAACATTAGA AGTGACGGAT 4700
4701 CAAATCATCA TGTCTCGTAA TTTTATTGTT AAATATGAAG TACGGGGCAA   AAATTGCTTA TTGCATCAAA ACCGACATTG GCCTTATTGA AGTGACGGAT 4800
4801 CTGATAGGTA TTTATTGTTA AGATCAAATT ATCTCTTGAA TCAGCTTTTA   GGTACAATTGA TGAATAACAA AAATGAATCA GACAAAAAGG ACGGCAGGTAG 4900
4901 AGAAGACAGG ACTCAAGACT TTGTTCTTGG GTACAATTGA TGAATAACAA   AGATAATAAG CTTTCTGACA CAAGAGAAAA ACTGAAAAA  TTAGCGAAAT 5000
5001 CTACATAACA TAATAAAACA GCTACAAGAA ATAGAACTAA AAAACTCAGT   AGATAATAAG CTTTCTGACA CAAGAGAAAA ACTGAAAAA  AACTTGATAT 5100
5101 TATCCTATAT GGAGAATGAGC AAAACTCCTG ATATAAAAAT ATATGTGCTG   AAGCACTAA  TCAAGTGGT  CCCAAATTCA TCACCATAGA 5200
5201 TTTATCAGCA GGAACTGATA ATGAATGGCTTG CAAGACAGTG TAAGCATGGT   CCCAAATTCA TCACCATAGA 5300
5301 GGCAGATGAT ATGATATGGA CACCAAAGA  ATTAAAAGAG ACACTGTCTG   AAAATCACAC ACCAATATTT ACAGTTGTTA TTTAGAAAAT 5400
5401 ATAGAAATAA TATATGTTAA AGCTTACTTA AGTTAGTAAA AAATAAATAG   ATGGGATAGC AAACATTAGA TGTCATCAAA AGTGACGGAT 5500
5501 CCTCAGAAAC ATGTAATCAA CTCAACAAAA TAATAAAAAA ACACTCAGGT   AAATTGCTTA TTGCATCAAA ACCGACATTG GCCTTATTGA AGTGACGGAT 5600
5601 AGTAACAATT ACTGTGTCAA ATACAAAAGT AGAAAATAAT TTGCAGGCAT   GTCAATTAAA AAATGAATCA GACAAAAAGG ACACCACCA  AATATACCACA 5700
5701 TCAACAACAA TCAGACACCAT TCCTGATCTA AATGCAGTAC AATGCAGTGAA   AAGGCTGATT CAGAAAAGAC CACAAAAGAC AGAGATACCT 5800
5801 GTTGGAGAAT CAACACCGAA CAATGCACAA ATATAAAAAT ATATAAGTTC   TTATGTTTTG GGTCTATGGA TTCAACAAAT ACAGACGAGT 5900
5901 AGTTCTATGC AGTTTTCAAC AGTTTTTCAC CACCCCAAA  CAATAGGAAAG   CAGAGTGTCA CCGTCCACAT ACAACCGAGT GGTGGTGCCA 6000
6001 GAGAGAAACTC AGTTTTCAAC AGTTTTAAAA GACAAAAAA  TATCTTTAA   TATTAATATA TCACTGAGTA CTCTAAATAT TGTCTAGACT 6100
6101 TCACAACACC CTGCGGTCAT ATGGGTCAA  CAATGGTCAA ACCACTGTTG   TATAATACAA TCACTGAGTA GTAGCAAGTG CCATAGAAAT 6200
6201 ACAAGTGGCC ATGGAAGCAA GAGTGGAGAA CATTCGGGCA ATAGACATGT   GATGAAAAAC CAACATCAAA AAACATGACC AAAGTGGAAC 6300
6301 GCTACACTGA TCCTTATTGG ATCAACAGCA CCAAGTATGG CACTCAACAC   CCTTTAATC  ATTGATCATG CCCAAACAAC CCAGAGCT   CAACACAGCT 6400
6401 ACTGTGTCAA CATGCCGCCG GTAGAACCAA GCAAGAAGAC CCCAAATGAA   TCTGCAGCAG ACCCAAACAC CCAGATGCAA GTCAGACTTA 6500
6501 GACCAGAGAG GATTCAACAT CTCTAGCAGC AACCCTAGAG GACCATCTAC   ACACAGGGAC AACTCCAACA CCGAGAGCAA GCAAACCACA 6600
6601 GACGAGCCAC CAACAACTGCT GAGATCAACC AACAGACAGA CCACCCAAAC   AACCCAGAGG AAAAAGCCAA CCAGAGCAAC GAAACCACAA 6700
6701 CTCGAACCAC CAAGACCAGC GCAACCCAAA CACTCAACAC CACCCAACCA   ACTAGCCAATG GAAGAGAGGC AACCACAACA TCTGCCCAGAT 6800
6801 TGCCACAACT CAAGACCAGCG ATCAGACAAC CCAGGCAGCA GACCCAAGCT   CCCAATCACA AAAAGCACAG CAACACCACA CAAACCAGAC 6900
6901 ACATCTTCTC CAAGTAGTTA ACAAAAAAAC TATAAAATAA CCATGAAAAC   AATAATACCA TTTGAACTCA GAAAATAAGA AGAAGTAAGA 7000
7001 ATGAATTGTT TGAGCGTATA TACTAATGAA ATAGCATCTG TTTGTGCATC   AATAATACCA TCATTATTTA AGAAATAAGA ATTCAAGGGA 7100
7101 CAAATAACAA TGGATCCGTT TTGTGAATCC ACTGTCAATG TCTATCTTCC   TGATTCATAT CTCAAAGGAG TAATATCTTT CAGTGAAAC  AATGCAATTG 7200
7201 GCTCATGCCT TTTGAAAAGA CCCTATCTTA AAAAAGATAA CACTGCTAAA   GTTGCTGTAG TGTTGAACAT GTCAGACTTA GTCAGCAGT  GTCAGAGCGT 7300
7301 CATGACCAAA ATGAAGATAT CAGATTATAA AGTGGTTGAA TGCAGCATGA   TGCAGCATGA AATATACACA AATAATGAAA CACATTATTA CACATTATTA 7400
7401 AAACAATTCT TAACAAGAAG TAAAAACATT AGTCCCCTAA AATTAAGTAT   GATATGTGAT TGGTTACAGT TAAAATCCAC ACATCAATTC 7500
7501 TTAATTTTAT AGATGTGGAG TTTATACCCG TTTGGGTGAG CAATTGGTTT   AGTAACTGGT ATAATCTCAA TAAATTAATC TTAGAGTTTA 7600
7601 AGTAATAAGA ACTGGTTCAA TTTTATGCAG ATCACTAGGC AAGTTAGTTT   TCAATTGTATC ATCTTATGGG TGTGTAGTAA AAAGCAACAA 7700
7701 GTAAGTTTTT TCACATATAA CCAACTGTTA ACATGCAGTTA ATGTGATGTT   AAGTAGGTTC AATGCAAACT TTTGTATATG GGTAAGTAAC 7800
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Fig. 39B

HMPV strain 75 (continued)

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7801  AAAATCAAGA AGGACTAGGA TTTAGAAGTA ATCTACAAGG TATGTTAACT   AATAAAATTAT ATGAAACTGT TGATTATATG TTAAGTCTAT GTAGCAATGA  7900
7901  AGGGTTCTCA CTAGTGAAAG AGTTCGAAGG CTTTATTATG AGTGAAATTC   TTAAAATTAC TGAGCATGCT CAATTCAGTA CTAGGTTTAG GAATACTTTA  8000
8001  TTAAATGGGT TGACTGAACA ATTATCAATG TTGAAAGCTA AAAACAGATC   TAGAGTTCTT GGCACTATAT TAGAAAACAA TGATTACCCC ATGTATGAAG  8100
8101  TAGTACTTAA ATTATTAGGG GACACTTTGA AAAGTATAAA ATTATTAATT   AACAAGAATT TAGAAAATGC TGCAGAATTA TATTATATAT TCAGAAATTT  8200
8201  TGGACACCCT ATGGTAGATG AGAGGGAAGC AATGGATGCT GTTAAATTAA   ATAATGAGAT TACAAAAAAT CTTAAACTGG AGAGCTTAAC AGAACTAAGA  8300
8301  GGAGCATTTA TACTAAGAAT TATAAAAGGG TTTGTAGATA ATAATAAAAG   ATGGCCTAAA ATTAAGAATT TAAAAGTGCT CAGTAAAAGA TGGGTTATGT  8400
8401  ATTTCAAAGC TAAAAGTTAC CCTAGCCAAC TTGAGCTAAG TGTACAAGAT   TTTTTAGAAC ACAATTCGAA CAGGAATTTT CTGTCCCTGA  8500
8501  AAAAACCAAT CTTGAGATGG TATTAAATGA TAAAGCAATA TCTCCACCAA   AAAAGTTAAT ATGGTCGGTA ACAATTCGAA ATTATCTACC TGAAATTATA  8600
8601  AAAAATCAAT ATTTAGAAGA GGTCTTCAAT GCAAGTGACA GTCAAAGAAC   GAGGAGAGTC TTAGAATTTT ACTTAAAAGA TTGCAAATTT GATCAAAAAG  8700
8701  ACCTCAAACG TTATGTAACT AAACAAGAGT ATCTAAATGA CAAAGACCAC   ATTGTCTCAT TAACTGGGAA AGAAAAGAGAA TTAAGTGTAG GCAGGATGTT  8800
8801  TGCAATGCAA CCTGGCAAAC AAAGACAAAT ACAGATACTA GCGCAGAAAC   TTTTAGCTGA TAATGTGTTA CCCTTTTTCC CAGAAAACTT AACAAAGTAT  8900
8901  GGTGACTTGG ATCTCCAAAG AATTATGGAA ATGAAATCAG AGGAAGAATG   CATTAAAAACT ATAGTTACAA CAATTATATT GCAAGAGCCT CGCAAAGCTT  9000
9001  CCATAGTAAC AGACCTAAGT AAATTCAATC AAGCCTTTAG ACAGCTATCT   GCGCAGACGT AGCGAGATGAG TTACATGGCA CGCAAAGCTT  9100
9101  ATTTTGTTGG TTACATCTTA TTGTTCCCAT GACCACAATG ATATGTGCAT   ACAGACAATGC ACCACCAGAA ACAAAGGGGG AGTATGATAT AGACAAAATA  9200
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Fig. 39C

HMPV strain 75 (continued)

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Fig. 39D

SEQUENCE LISTING

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REPRESENTED BY THE SECRETARY OF THE DEPARTMENT OF HEALTH AND
HUMAN SERVICES
Collins, Peter L.
Biacchesi, Stephane
Buchholz, Ursula
Skiadopoulos, Mario H.
Murphy, Brian R.

<120> RECOMBINANT HUMAN METAPNEUMOVIRUS AND ITS USE

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<150> US 60/451,119
<151> 2003-02-28

<150> US 60/478,667
<151> 2003-06-13

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<170> PatentIn version 3.2

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caagaagagt actagagtac tatttaaaag ataataaatt tgatcaaaag gaacttaaaa     9480

gttatgtagt tagacaagaa tatttaaacg ataaggagca cattgtctca ttaactggaa     9540

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tacaaatatt ggcagaaaaa ttgttagctg ataacattgt acctttcttc ccggaaacct     9660
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21

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atgtcatata tgcaactagt tctcatttga aagggataat tatagaaaag ttcagcactg   11400

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22

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<211> 187
<212> PRT
<213> human metapneumovirus

<400> 4

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Arg Gly Ser Glu Cys Lys Phe Asn His Asn Tyr Trp Ser Trp Pro Asp
            20                  25                  30


Arg Tyr Leu Leu Ile Arg Ser Asn Tyr Leu Leu Asn Gln Leu Leu Arg
        35                  40                  45


Asn Thr Asp Arg Ala Asp Gly Leu Ser Ile Ile Ser Gly Ala Gly Arg
    50                  55                  60


Glu Asp Arg Thr Gln Asp Phe Val Leu Gly Ser Thr Asn Val Val Gln
65                  70                  75                  80


Gly Tyr Ile Asp Asp Asn Gln Ser Ile Thr Lys Ala Ala Ala Cys Tyr
                85                  90                  95
```

24

```
Ser Leu His Asn Ile Ile Lys Gln Leu Gln Glu Val Glu Val Arg Gln
            100               105           110


Ala Arg Asp Ser Lys Leu Ser Asp Ser Lys His Val Ala Leu His Asn
            115               120           125


Leu Ile Leu Ser Tyr Met Glu Met Ser Lys Thr Pro Ala Ser Leu Ile
            130               135           140


Asn Asn Leu Lys Arg Leu Pro Arg Glu Lys Leu Lys Lys Leu Ala Lys
145               150               155               160


Leu Ile Ile Asp Leu Ser Ala Gly Ala Asp Asn Asp Ser Ser Tyr Ala
                  165               170               175


Leu Gln Asp Ser Glu Ser Ile Asn Gln Val Gln
            180               185
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<210>   5
<211>   179
<212>   PRT
<213>   human metapneumovirus

<400>   5

Met Ile Thr Leu Asp Val Ile Lys Ser Asp Gly Ser Ser Lys Thr Cys
1               5               10              15


Thr His Leu Lys Lys Ile Ile Lys Asp His Ser Gly Lys Val Leu Ile
            20              25              30


Ala Leu Lys Leu Ile Leu Ala Leu Leu Thr Phe Phe Thr Ile Thr Ile
            35              40              45


Thr Ile Asn Tyr Ile Lys Val Glu Asn Asn Leu Gln Ile Cys Gln Ser
     50              55              60


Lys Thr Glu Ser Asp Lys Glu Asp Ser Pro Ser Asn Thr Thr Ser Val
65              70              75              80


Thr Thr Lys Thr Thr Leu Asp His Asp Ile Thr Gln Tyr Phe Lys Arg
            85              90              95


Leu Ile Gln Arg Tyr Thr Asp Ser Val Ile Asn Lys Asp Thr Cys Trp
            100               105           110


Lys Ile Ser Arg Asn Gln Cys Thr Asn Ile Thr Thr Tyr Lys Phe Leu
```

25

```
              115                      120                          125
```

Cys Phe Lys Pro Glu Asp Ser Lys Ile Asn Ser Cys Asp Arg Leu Thr
    130   135   140

Asp Leu Cys Arg Asn Lys Ser Lys Ser Ala Ala Glu Ala Tyr His Thr
145   150   155   160

Val Glu Cys His Cys Ile Tyr Thr Ile Glu Trp Lys Cys Tyr His His
                165   170   175

Ser Ile Asp

<210> 6
<211> 219
<212> PRT
<213> human metapneumovirus

<400> 6

Met Glu Val Lys Val Glu Asn Ile Arg Ala Ile Asp Met Leu Lys Ala
1   5   10   15

Arg Val Lys Asn Arg Val Ala Arg Ser Lys Cys Phe Lys Asn Ala Ser
            20   25   30

Leu Ile Leu Ile Gly Ile Thr Thr Leu Ser Ile Ala Leu Asn Ile Tyr
            35   40   45

Leu Ile Ile Asn Tyr Thr Ile Gln Lys Thr Ser Ser Glu Ser Glu His
        50   55   60

His Thr Ser Ser Pro Pro Thr Glu Ser Asn Lys Glu Ala Ser Thr Ile
65   70   75   80

Ser Thr Asp Asn Pro Asp Ile Asn Pro Asn Ser Gln His Pro Thr Gln
                85   90   95

Gln Ser Thr Glu Asn Pro Thr Leu Asn Pro Ala Ala Ser Val Ser Pro
            100   105   110

Ser Glu Thr Glu Pro Ala Ser Thr Pro Asp Thr Thr Asn Arg Leu Ser
            115   120   125

Ser Val Asp Arg Ser Thr Ala Gln Pro Ser Glu Ser Arg Thr Lys Thr
    130   135   140

                                    26

Lys Pro Thr Val His Thr Arg Asn Asn Pro Ser Thr Ala Ser Ser Thr
145   150   155   160

Gln Ser Pro Pro Arg Ala Thr Thr Lys Ala Ile Arg Arg Ala Thr Thr
                165   170   175

Phe Arg Met Ser Ser Thr Gly Lys Arg Pro Thr Thr Thr Ser Val Gln
            180   185   190

Ser Asp Ser Ser Thr Thr Thr Gln Asn His Glu Glu Thr Gly Ser Ala
            195   200   205

Asn Pro Gln Ala Ser Val Ser Thr Met Gln Asn
    210   215

<210> 7
<211> 2005
<212> PRT
<213> human metapneumovirus

<400> 7

Met Asp Pro Leu Asn Glu Ser Thr Val Asn Val Tyr Leu Pro Asp Ser
1   5   10   15

Tyr Leu Lys Gly Val Ile Ser Phe Ser Glu Thr Asn Ala Ile Gly Ser
            20   25   30

Cys Leu Leu Lys Arg Pro Tyr Leu Lys Asn Asp Asn Thr Ala Lys Val
            35   40   45

Ala Ile Glu Asn Pro Val Ile Glu His Val Arg Leu Lys Asn Ala Val
    50   55   60

Asn Ser Lys Met Lys Ile Ser Asp Tyr Lys Val Val Glu Pro Val Asn
65   70   75   80

Met Gln His Glu Ile Met Lys Asn Val His Ser Cys Glu Leu Thr Leu
            85   90   95

Leu Lys Gln Phe Leu Thr Arg Ser Lys Asn Ile Ser Thr Leu Lys Leu
            100   105   110

Asn Met Ile Cys Asp Trp Leu Gln Leu Lys Ser Thr Ser Asp Asp Thr
            115   120   125

27

Ser Ile Leu Ser Phe Ile Asp Val Glu Phe Ile Pro Ser Trp Val Ser
    130   135   140

Asn Trp Phe Ser Asn Trp Tyr Asn Leu Asn Lys Leu Ile Leu Glu Phe
145   150   155   160

Arg Arg Glu Glu Val Ile Arg Thr Gly Ser Ile Leu Cys Arg Ser Leu
            165   170   175

Gly Lys Leu Val Phe Ile Val Ser Ser Tyr Gly Cys Ile Val Lys Ser
            180   185   190

Asn Lys Ser Lys Arg Val Ser Phe Phe Thr Tyr Asn Gln Leu Leu Thr
        195   200   205

Trp Lys Asp Val Met Leu Ser Arg Phe Asn Ala Asn Phe Cys Ile Trp
    210   215   220

Val Ser Asn Ser Leu Asn Glu Asn Gln Glu Gly Leu Gly Leu Arg Ser
225   230   235   240

Asn Leu Gln Gly Met Leu Thr Asn Lys Leu Tyr Glu Thr Val Asp Tyr
            245   250   255

Met Leu Ser Leu Cys Cys Asn Glu Gly Phe Ser Leu Val Lys Glu Phe
            260   265   270

Glu Gly Phe Ile Met Ser Glu Ile Leu Arg Ile Thr Glu His Ala Gln
            275   280   285

Phe Ser Thr Arg Phe Arg Asn Thr Leu Leu Asn Gly Leu Thr Asp Gln
    290   295   300

Leu Thr Lys Leu Lys Asn Lys Asn Arg Leu Arg Val His Ser Thr Val
305   310   315   320

Leu Glu Asn Asn Asp Tyr Pro Met Tyr Glu Val Val Leu Lys Leu Leu
            325   330   335

Gly Asp Thr Leu Arg Cys Ile Lys Leu Leu Ile Asn Lys Asn Leu Glu
            340   345   350

Asn Ala Ala Glu Leu Tyr Tyr Ile Phe Arg Ile Phe Gly His Pro Met
        355   360   365

Val Asp Glu Arg Asp Ala Met Asp Ala Val Lys Leu Asn Asn Glu Ile
    370   375   380

Thr Lys Ile Leu Arg Leu Glu Ser Leu Thr Glu Leu Arg Gly Ala Phe
385   390   395   400

Ile Leu Arg Ile Ile Lys Gly Phe Val Asp Asn Asn Lys Arg Trp Pro
                405   410   415

Lys Ile Lys Asn Leu Lys Val Leu Ser Lys Arg Trp Thr Met Tyr Phe
                420   425   430

Lys Ala Lys Asn Tyr Pro Ser Gln Leu Glu Leu Ser Glu Gln Asp Phe
            435   440   445

Leu Glu Leu Ala Ala Ile Gln Phe Glu Gln Glu Phe Ser Val Pro Glu
    450   455   460

Lys Thr Asn Leu Glu Met Val Leu Asn Asp Lys Ala Ile Ser Pro Pro
465   470   475   480

Lys Arg Leu Ile Trp Ser Val Tyr Pro Lys Asn Tyr Leu Pro Glu Thr
                485   490   495

Ile Lys Asn Arg Tyr Leu Glu Glu Thr Phe Asn Ala Ser Asp Ser Leu
            500   505   510

Lys Thr Arg Arg Val Leu Glu Tyr Tyr Leu Lys Asp Asn Lys Phe Asp
            515   520   525

Gln Lys Glu Leu Lys Ser Tyr Val Val Arg Gln Glu Tyr Leu Asn Asp
    530   535   540

Lys Glu His Ile Val Ser Leu Thr Gly Lys Glu Arg Glu Leu Ser Val
545   550   555   560

Gly Arg Met Phe Ala Met Gln Pro Gly Lys Gln Arg Gln Ile Gln Ile
            565   570   575

Leu Ala Glu Lys Leu Leu Ala Asp Asn Ile Val Pro Phe Phe Pro Glu
            580   585   590

Thr Leu Thr Lys Tyr Gly Asp Leu Asp Leu Gln Arg Ile Met Glu Ile
        595   600   605

29

Lys Ser Glu Leu Ser Ser Ile Lys Thr Arg Arg Asn Asp Ser Tyr Asn
    610   615   620

Asn Tyr Ile Ala Arg Ala Ser Ile Val Thr Asp Leu Ser Lys Phe Asn
625   630   635   640

Gln Ala Phe Arg Tyr Glu Thr Thr Ala Ile Cys Ala Asp Val Ala Asp
            645   650   655

Glu Leu His Gly Thr Gln Ser Leu Phe Cys Trp Leu His Leu Ile Val
            660   665   670

Pro Met Thr Thr Met Ile Cys Ala Tyr Arg His Ala Pro Pro Glu Thr
        675   680   685

Lys Gly Glu Tyr Asp Ile Asp Lys Ile Glu Glu Gln Ser Gly Leu Tyr
    690   695   700

Arg Tyr His Met Gly Gly Ile Glu Gly Trp Cys Gln Lys Leu Trp Thr
705   710   715   720

Met Glu Ala Ile Ser Leu Leu Asp Val Val Ser Val Lys Thr Arg Cys
            725   730   735

Gln Met Thr Ser Leu Leu Asn Gly Asp Asn Gln Ser Ile Asp Val Ser
            740   745   750

Lys Pro Val Lys Leu Ser Glu Gly Leu Asp Glu Val Lys Ala Asp Tyr
    755   760   765

Arg Leu Ala Val Lys Met Leu Lys Glu Ile Arg Asp Ala Tyr Arg Asn
    770   775   780

Ile Gly His Lys Leu Lys Glu Gly Glu Thr Tyr Ile Ser Arg Asp Leu
785   790   795   800

Gln Phe Ile Ser Lys Val Ile Gln Ser Glu Gly Val Met His Pro Thr
            805   810   815

Pro Ile Lys Lys Val Leu Arg Val Gly Pro Trp Ile Asn Thr Ile Leu
            820   825   830

Asp Asp Ile Lys Thr Ser Ala Glu Ser Ile Gly Ser Leu Cys Gln Glu
        835   840   845

30

Leu Glu Phe Arg Gly Glu Ser Ile Ile Val Ser Leu Ile Leu Arg Asn
    850   855   860

Phe Trp Leu Tyr Asn Leu Tyr Met His Glu Ser Lys Gln His Pro Leu
865   870   875   880

Ala Gly Lys Gln Leu Phe Lys Gln Leu Asn Lys Thr Leu Thr Ser Val
            885   890   895

Gln Arg Phe Phe Glu Ile Lys Arg Glu Asn Glu Val Val Asp Leu Trp
        900   905   910

Met Asn Ile Pro Met Gln Phe Gly Gly Gly Asp Pro Val Val Phe Tyr
        915   920   925

Arg Ser Phe Tyr Arg Arg Thr Pro Asp Phe Leu Thr Glu Ala Ile Ser
    930   935   940

His Val Asp Ile Leu Leu Lys Ile Ser Ala Asn Ile Lys Asn Glu Thr
945   950   955   960

Lys Val Ser Phe Phe Lys Ala Leu Leu Ser Ile Glu Lys Asn Glu Arg
            965   970   975

Ala Thr Leu Thr Thr Leu Met Arg Asp Pro Gln Ala Val Gly Ser Glu
        980   985   990

Arg Gln Ala Lys Val Thr Ser Asp Ile Asn Arg Thr Ala Val Thr Ser
        995   1000   1005

Ile Leu Ser Leu Ser Pro Asn Gln Leu Phe Ser Asp Ser Ala Ile
    1010   1015   1020

His Tyr Ser Arg Asn Glu Glu Glu Val Gly Ile Ile Ala Glu Asn
    1025   1030   1035

Ile Thr Pro Val Tyr Pro His Gly Leu Arg Val Leu Tyr Glu Ser
    1040   1045   1050

Leu Pro Phe His Lys Ala Glu Lys Val Val Asn Met Ile Ser Gly
    1055   1060   1065

Thr Lys Ser Ile Thr Asn Leu Leu Gln Arg Thr Ser Ala Ile Asn
    1070   1075   1080

31

Gly Glu Asp Ile Asp Arg Ala   Val Ser Met Met Leu   Glu Asn Leu
    1085   1090   1095

Gly Leu Leu Ser Arg Ile Leu   Ser Val Val Val Asp   Ser Ile Glu
    1100   1105   1110

Ile Pro Ile Lys Ser Asn Gly   Arg Leu Ile Cys Cys   Gln Ile Ser
    1115   1120   1125

Arg Thr Leu Arg Glu Thr Ser   Trp Asn Asn Met Glu   Ile Val Gly
    1130   1135   1140

Val Thr Ser Pro Ser Ile Thr   Thr Cys Met Asp Val   Ile Tyr Ala
    1145   1150   1155

Thr Ser Ser His Leu Lys Gly   Ile Ile Ile Glu Lys   Phe Ser Thr
    1160   1165   1170

Asp Arg Thr Thr Arg Gly Gln   Arg Gly Pro Lys Ser   Pro Trp Val
    1175   1180   1185

Gly Ser Ser Thr Gln Glu Lys   Lys Leu Val Pro Val   Tyr Asn Arg
    1190   1195   1200

Gln Ile Leu Ser Lys Gln Gln   Arg Glu Gln Leu Glu   Ala Ile Gly
    1205   1210   1215

Lys Met Arg Trp Val Tyr Lys   Gly Thr Pro Gly Leu   Arg Arg Leu
    1220   1225   1230

Leu Asn Lys Ile Cys Leu Gly   Ser Leu Gly Ile Ser   Tyr Lys Cys
    1235   1240   1245

Val Lys Pro Leu Leu Pro Arg   Phe Met Ser Val Asn   Phe Leu His
    1250   1255   1260

Arg Leu Ser Val Ser Ser Arg   Pro Met Glu Phe Pro   Ala Ser Val
    1265   1270   1275

Pro Ala Tyr Arg Thr Thr Asn   Tyr His Phe Asp Thr   Ser Pro Ile
    1280   1285   1290

Asn Gln Ala Leu Ser Glu Arg   Phe Gly Asn Glu Asp   Ile Asn Leu
    1295   1300   1305

32

Val Phe Gln Asn Ala Ile Ser Cys Gly Ile Ser Ile Met Ser Val
1310   1315   1320

Val Glu Gln Leu Thr Gly Arg Ser Pro Lys Gln Leu Val Leu Ile
1325   1330   1335

Pro Gln Leu Glu Glu Ile Asp Ile Met Pro Pro Pro Val Phe Gln
1340   1345   1350

Gly Lys Phe Asn Tyr Lys Leu Val Asp Lys Ile Thr Ser Asp Gln
1355   1360   1365

His Ile Phe Ser Pro Asp Lys Ile Asp Met Leu Thr Leu Gly Lys
1370   1375   1380

Met Leu Met Pro Thr Ile Lys Gly Gln Lys Thr Asp Gln Phe Leu
1385   1390   1395

Asn Lys Arg Glu Asn Tyr Phe His Gly Asn Asn Leu Ile Glu Ser
1400   1405   1410

Leu Ser Ala Ala Leu Ala Cys His Trp Cys Gly Ile Leu Thr Glu
1415   1420   1425

Gln Cys Ile Glu Asn Asn Ile Phe Lys Lys Asp Trp Gly Asp Gly
1430   1435   1440

Phe Ile Ser Asp His Ala Phe Met Asp Phe Lys Ile Phe Leu Cys
1445   1450   1455

Val Phe Lys Thr Lys Leu Leu Cys Ser Trp Gly Ser Gln Gly Lys
1460   1465   1470

Asn Ile Lys Asp Glu Asp Ile Val Asp Glu Ser Ile Asp Lys Leu
1475   1480   1485

Leu Arg Ile Asp Asn Thr Phe Trp Arg Met Phe Ser Lys Val Met
1490   1495   1500

Phe Glu Pro Lys Val Lys Lys Arg Ile Met Leu Tyr Asp Val Lys
1505   1510   1515

Phe Leu Ser Leu Val Gly Tyr Ile Gly Phe Lys Asn Trp Phe Ile
1520   1525   1530

33

Glu Gln Leu Arg Ser Ala Glu Leu His Glu Ile Pro Trp Ile Val
    1535   1540   1545

Asn Ala Glu Gly Asp Leu Val Glu Ile Lys Ser Ile Lys Ile Tyr
    1550   1555   1560

Leu Gln Leu Ile Glu Gln Ser Leu Phe Leu Arg Ile Thr Val Leu
    1565   1570   1575

Asn Tyr Thr Asp Met Ala His Ala Leu Thr Arg Leu Ile Arg Lys
    1580   1585   1590

Lys Leu Met Cys Asp Asn Ala Leu Leu Thr Pro Ile Ser Ser Pro
    1595   1600   1605

Met Val Asn Leu Thr Gln Val Ile Asp Pro Thr Thr Gln Leu Asp
    1610   1615   1620

Tyr Phe Pro Lys Ile Thr Phe Glu Arg Leu Lys Asn Tyr Asp Thr
    1625   1630   1635

Ser Ser Asn Tyr Ala Lys Gly Lys Leu Thr Arg Asn Tyr Met Ile
    1640   1645   1650

Leu Leu Pro Trp Gln His Val Asn Arg Tyr Asn Phe Val Phe Ser
    1655   1660   1665

Ser Thr Gly Cys Lys Val Ser Leu Lys Thr Cys Ile Gly Lys Leu
    1670   1675   1680

Met Lys Asp Leu Asn Pro Lys Val Leu Tyr Phe Ile Gly Glu Gly
    1685   1690   1695

Ala Gly Asn Trp Met Ala Arg Thr Ala Cys Glu Tyr Pro Asp Ile
    1700   1705   1710

Lys Phe Val Tyr Arg Ser Leu Lys Asp Asp Leu Asp His His Tyr
    1715   1720   1725

Pro Leu Glu Tyr Gln Arg Val Ile Gly Glu Leu Ser Arg Ile Ile
    1730   1735   1740

Asp Ser Gly Glu Gly Leu Ser Met Glu Thr Thr Asp Ala Thr Gln
    1745   1750   1755

34

Lys Thr His Trp Asp Leu Ile His Arg Val Ser Lys Asp Ala Leu
    1760   1765   1770

Leu Ile Thr Leu Cys Asp Ala Glu Phe Lys Asp Arg Asp Asp Phe
    1775   1780   1785

Phe Lys Met Val Ile Leu Trp Arg Lys His Val Leu Ser Cys Arg
    1790   1795   1800

Ile Cys Thr Thr Tyr Gly Thr Asp Leu Tyr Leu Phe Ala Lys Tyr
    1805   1810   1815

His Ala Lys Asp Cys Asn Val Lys Leu Pro Phe Phe Val Arg Ser
    1820   1825   1830

Val Ala Thr Phe Ile Met Gln Gly Ser Lys Leu Ser Gly Ser Glu
    1835   1840   1845

Cys Tyr Ile Leu Leu Thr Leu Gly His His Asn Ser Leu Pro Cys
    1850   1855   1860

His Gly Glu Ile Gln Asn Ser Lys Met Lys Ile Ala Val Cys Asn
    1865   1870   1875

Asp Phe Tyr Ala Ala Lys Lys Leu Asp Asn Lys Ser Ile Glu Ala
    1880   1885   1890

Asn Cys Lys Ser Leu Leu Ser Gly Leu Arg Ile Pro Ile Asn Lys
    1895   1900   1905

Lys Glu Leu Asp Arg Gln Arg Arg Leu Leu Thr Leu Gln Ser Asn
    1910   1915   1920

His Ser Ser Val Ala Thr Val Gly Gly Ser Lys Ile Ile Glu Ser
    1925   1930   1935

Lys Trp Leu Thr Asn Lys Ala Ser Thr Ile Ile Asp Trp Leu Glu
    1940   1945   1950

His Ile Leu Asn Ser Pro Lys Gly Glu Leu Asn Tyr Asp Phe Phe
    1955   1960   1965

Glu Ala Leu Glu Asn Thr Tyr Pro Asn Met Ile Lys Leu Ile Asp
    1970   1975   1980

Asn Leu Gly Asn Ala Glu Ile Lys Lys Leu Ile Lys Val Thr Gly
    1985   1990   1995

Tyr Met Leu Val Ser Lys Lys
    2000   2005

<210>   8
<211>   177
<212>   PRT
<213>   human metapneumovirus

<400>   8

Met Lys Thr Leu Asp Val Ile Lys Ser Asp Gly Ser Ser Glu Thr Cys
1   5   10   15

Asn Gln Leu Lys Lys Ile Ile Lys Lys His Ser Gly Lys Leu Leu Ile
            20   25   30

Ala Ser Lys Pro Thr Leu Ala Leu Leu Thr Ser Phe Thr Val Thr Ile
            35   40   45

Thr Val Asn Tyr Thr Lys Val Glu Asn Asn Leu Gln Ala Cys Gln Leu
    50   55   60

Lys Asn Glu Ser Asp Lys Lys Asp Thr Lys Leu Asn Thr Thr Ser Thr
65   70   75   80

Thr Ile Arg Pro Ile Pro Asp Leu Asn Ala Val Gln Tyr Leu Lys Arg
                85   90   95

Leu Ile Gln Lys His Thr Asn Ser Val Thr Lys Asp Arg Asp Thr Cys
            100   105   110

Trp Arg Ile His Thr Asn Gln Cys Thr Asn Ile Lys Ile Tyr Lys Phe
            115   120   125

Leu Cys Phe Gly Ser Met Asn Ser Thr Asn Thr Asp Cys Glu Glu Pro
    130   135   140

Thr Val Leu Cys Asp Lys Lys Ser Lys Thr Met Thr Glu Lys His Arg
145   150   155   160

Lys Ala Glu Cys His Arg Pro His Thr Thr Glu Trp Trp Cys His Tyr
                165   170   175

Leu

<210>   9
<211>   236
<212>   PRT
<213>   human metapneumovirus

<400>   9

Met Glu Ala Arg Val Glu Asn Ile Arg Ala Ile Asp Met Phe Lys Ala
1   5   10   15

Lys Met Lys Asn Arg Ile Arg Ser Ser Lys Cys His Arg Asn Ala Thr
            20   25   30

Leu Ile Leu Ile Gly Ser Thr Ala Pro Ser Met Ala Leu Asn Thr Leu
            35   40   45

Leu Ile Ile Asp His Ala Thr Ser Lys Asn Met Thr Lys Val Glu His
        50   55   60

Cys Val Asn Met Pro Pro Val Glu Pro Ser Lys Lys Thr Pro Met Thr
65   70   75   80

Ser Ala Ala Asp Pro Asn Thr Lys Pro Asn Pro Gln Gln Ala Thr Gln
                85   90   95

Leu Thr Thr Glu Asp Ser Thr Ser Leu Ala Ala Thr Leu Glu Asp His
            100   105   110

Leu His Thr Gly Thr Thr Pro Thr Pro Asp Ala Thr Val Ser Gln Gln
        115   120   125

Thr Thr Asp Glu His Thr Thr Leu Leu Arg Ser Thr Asn Arg Gln Thr
        130   135   140

Thr Gln Thr Thr Ala Glu Lys Lys Pro Thr Arg Ala Thr Thr Lys Lys
145   150   155   160

Glu Thr Thr Thr Arg Thr Thr Ser Thr Ala Ala Thr Gln Thr Leu Asn
                165   170   175

Thr Thr Asn Gln Thr Ser Asn Gly Arg Glu Ala Thr Thr Thr Ser Ala
            180   185   190

Arg Ser Arg Asn Asn Ala Thr Thr Gln Ser Ser Asp Gln Thr Thr Gln

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              195                    200                      205


Ala Ala Asp Pro Ser Ser Gln Ser Gln His Thr Gln Lys Ser Thr Thr
    210                 215                 220


Thr Thr His Asn Thr Asp Thr Ser Ser Pro Ser Ser
225                 230                 235
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<210> 10
<211> 183
<212> PRT
<213> human metapneumovirus

<400> 10

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Met Ile Thr Leu Asp Val Ile Lys Ser Asp Gly Ser Ser Lys Thr Cys
1               5                10                  15


Thr His Leu Lys Lys Ile Ile Lys Asp His Ser Gly Lys Val Leu Ile
            20                  25                  30


Val Leu Lys Leu Ile Leu Ala Leu Leu Thr Phe Leu Thr Val Thr Ile
            35                  40                  45


Thr Ile Asn Tyr Ile Lys Val Glu Asn Asn Leu Gln Ile Cys Gln Ser
    50                  55                  60


Lys Thr Glu Ser Asp Lys Lys Asp Ser Ser Ser Asn Thr Thr Ser Val
65                  70                  75                  80


Thr Thr Lys Thr Thr Leu Asn His Asp Ile Thr Gln Tyr Phe Lys Ser
                85                  90                  95


Leu Ile Gln Arg Tyr Thr Asn Ser Ala Ile Asn Ser Asp Thr Cys Trp
            100                 105                 110


Lys Ile Asn Arg Asn Gln Cys Thr Asn Ile Thr Thr Tyr Lys Phe Leu
            115                 120                 125


Cys Phe Lys Ser Glu Asp Thr Lys Thr Asn Asn Cys Asp Lys Leu Thr
    130                 135                 140


Asp Leu Cys Arg Asn Lys Pro Lys Pro Ala Val Gly Val Tyr His Ile
145                 150                 155                 160


Val Glu Cys His Cys Ile Tyr Thr Val Lys Trp Lys Cys Tyr His Tyr
                165                 170                 175
```

Pro Thr Asp Glu Thr Gln Ser
            180

<210>   11
<211>   236
<212>   PRT
<213>   human metapneumovirus

<400>   11

Met Glu Val Lys Val Glu Asn Ile Arg Thr Ile Asp Met Leu Lys Ala
1   5   10   15

Arg Val Lys Asn Arg Val Ala Arg Ser Lys Cys Phe Lys Asn Ala Ser
            20   25   30

Leu Val Leu Ile Gly Ile Thr Thr Leu Ser Ile Ala Leu Asn Ile Tyr
            35   40   45

Leu Ile Ile Asn Tyr Lys Met Gln Lys Asn Thr Ser Glu Ser Glu His
        50   55   60

His Thr Ser Ser Ser Pro Met Glu Ser Ser Arg Glu Thr Pro Thr Val
65   70   75   80

Pro Thr Asp Asn Ser Asp Thr Asn Ser Ser Pro Gln His Pro Thr Gln
                85   90   95

Gln Ser Thr Glu Gly Ser Thr Leu Tyr Phe Ala Ala Ser Ala Ser Ser
            100   105   110

Pro Glu Thr Glu Pro Thr Ser Thr Pro Asp Thr Thr Asn Arg Pro Pro
            115   120   125

Phe Val Asp Thr His Thr Thr Pro Pro Ser Ala Ser Arg Thr Lys Thr
    130   135   140

Ser Pro Ala Val His Thr Lys Asn Asn Pro Arg Thr Ser Ser Arg Thr
145   150   155   160

His Ser Pro Pro Arg Ala Thr Thr Arg Thr Ala Arg Arg Thr Thr Thr
                165   170   175

Leu Arg Thr Ser Ser Thr Arg Lys Arg Pro Ser Thr Ala Ser Val Gln
            180   185   190

Pro Asp Ile Ser Ala Thr Thr His Lys Asn Glu Glu Ala Ser Pro Ala
    195   200   205

Ser Pro Gln Thr Ser Ala Ser Thr Thr Arg Ile Gln Arg Lys Ser Val
    210   215   220

Glu Ala Asn Thr Ser Thr Thr Tyr Asn Gln Thr Ser
225   230   235

<210> 12
<211> 2005
<212> PRT
<213> human metapneumovirus

<400> 12

Met Asp Pro Leu Asn Glu Ser Thr Val Asn Val Tyr Leu Pro Asp Ser
1   5   10   15

Tyr Leu Lys Gly Val Ile Ser Phe Ser Glu Thr Asn Ala Ile Gly Ser
            20   25   30

Cys Leu Leu Lys Arg Pro Tyr Leu Lys Asn Asp Asn Thr Ala Lys Val
        35   40   45

Ala Ile Glu Asn Pro Val Ile Glu His Val Arg Leu Lys Asn Ala Val
    50   55   60

Asn Ser Lys Met Lys Ile Ser Asp Tyr Lys Ile Val Glu Pro Val Asn
65   70   75   80

Met Gln His Glu Ile Met Lys Asn Val His Ser Cys Glu Leu Thr Leu
                85   90   95

Leu Lys Gln Phe Leu Thr Arg Ser Lys Asn Ile Ser Thr Leu Lys Leu
            100   105   110

Asn Met Ile Cys Asp Trp Leu Gln Leu Lys Ser Thr Ser Asp Asp Thr
        115   120   125

Ser Ile Leu Ser Phe Ile Asp Val Glu Phe Ile Pro Ser Trp Val Ser
    130   135   140

Asn Trp Phe Ser Asn Trp Tyr Asn Leu Asn Lys Leu Ile Leu Glu Phe
145   150   155   160

40

Arg Lys Glu Glu Val Ile Arg Thr Gly Ser Ile Leu Cys Arg Ser Leu
              165   170   175

Gly Lys Leu Val Phe Val Val Ser Ser Tyr Gly Cys Ile Val Lys Ser
              180   185   190

Asn Lys Ser Lys Arg Val Ser Phe Phe Thr Tyr Asn Gln Leu Leu Thr
              195   200   205

Trp Lys Asp Val Met Leu Ser Arg Phe Asn Ala Asn Phe Cys Ile Trp
      210   215   220

Val Ser Asn Ser Leu Asn Glu Asn Gln Glu Gly Leu Gly Leu Arg Ser
225   230   235   240

Asn Leu Gln Gly Ile Leu Thr Asn Lys Leu Tyr Glu Thr Val Asp Tyr
              245   250   255

Met Leu Ser Leu Cys Cys Asn Glu Gly Phe Ser Leu Val Lys Glu Phe
              260   265   270

Glu Gly Phe Ile Met Ser Glu Ile Leu Arg Ile Thr Glu His Ala Gln
              275   280   285

Phe Ser Thr Arg Phe Arg Asn Thr Leu Leu Asn Gly Leu Thr Asp Gln
      290   295   300

Leu Thr Lys Leu Lys Asn Lys Asn Arg Leu Arg Val His Gly Thr Val
305   310   315   320

Leu Glu Asn Asn Asp Tyr Pro Met Tyr Glu Val Val Leu Lys Leu Leu
              325   330   335

Gly Asp Thr Leu Arg Cys Ile Lys Leu Leu Ile Asn Lys Asn Leu Glu
              340   345   350

Asn Ala Ala Glu Leu Tyr Tyr Ile Phe Arg Ile Phe Gly His Pro Met
      355   360   365

Val Asp Glu Arg Asp Ala Met Asp Ala Val Lys Leu Asn Asn Glu Ile
      370   375   380

Thr Lys Ile Leu Arg Trp Glu Ser Leu Thr Glu Leu Arg Gly Ala Phe
385   390   395   400

41

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Ile Leu Arg Ile Ile Lys Gly Phe Val Asp Asn Asn Lys Arg Trp Pro
              405                 410                 415

Lys Ile Lys Asn Leu Lys Val Leu Ser Lys Arg Trp Thr Met Tyr Phe
              420                 425                 430

Lys Ala Lys Ser Tyr Pro Ser Gln Leu Glu Leu Ser Glu Gln Asp Phe
              435                 440                 445

Leu Glu Leu Ala Ala Ile Gln Phe Glu Gln Glu Phe Ser Val Pro Glu
    450                 455                 460

Lys Thr Asn Leu Glu Met Val Leu Asn Asp Lys Ala Ile Ser Pro Pro
465                 470                 475                 480

Lys Arg Leu Ile Trp Ser Val Tyr Pro Lys Asn Tyr Leu Pro Glu Lys
              485                 490                 495

Ile Lys Asn Arg Tyr Leu Glu Glu Thr Phe Asn Ala Ser Asp Ser Leu
              500                 505                 510

Lys Thr Arg Arg Val Leu Glu Tyr Tyr Leu Lys Asp Asn Lys Phe Asp
              515                 520                 525

Gln Lys Glu Leu Lys Ser Tyr Val Val Lys Gln Glu Tyr Leu Asn Asp
    530                 535                 540

Lys Asp His Ile Val Ser Leu Thr Gly Lys Glu Arg Glu Leu Ser Val
545                 550                 555                 560

Gly Arg Met Phe Ala Met Gln Pro Gly Lys Gln Arg Gln Ile Gln Ile
              565                 570                 575

Leu Ala Glu Lys Leu Leu Ala Asp Asn Ile Val Pro Phe Phe Pro Glu
              580                 585                 590

Thr Leu Thr Lys Tyr Gly Asp Leu Asp Leu Gln Arg Ile Met Glu Ile
              595                 600                 605

Lys Ser Glu Leu Ser Ser Ile Lys Thr Arg Arg Asn Asp Ser Tyr Asn
              610                 615                 620

Asn Tyr Ile Ala Arg Ala Ser Ile Val Thr Asp Leu Ser Lys Phe Asn
625                 630                 635                 640
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Gln Ala Phe Arg Tyr Glu Thr Thr Ala Ile Cys Ala Asp Val Ala Asp
            645                 650             655

Glu Leu His Gly Thr Gln Ser Leu Phe Cys Trp Leu His Leu Ile Val
            660                 665             670

Pro Met Thr Thr Met Ile Cys Ala Tyr Arg His Ala Pro Pro Glu Thr
            675                 680             685

Lys Gly Glu Tyr Asp Ile Asp Lys Ile Glu Glu Gln Ser Gly Leu Tyr
            690                 695             700

Arg Tyr His Met Gly Gly Ile Glu Gly Trp Cys Gln Lys Leu Trp Thr
705             710             715                 720

Met Glu Ala Ile Ser Leu Leu Asp Val Val Ser Val Lys Thr Arg Cys
                725             730             735

Gln Met Thr Ser Leu Leu Asn Gly Asp Asn Gln Ser Ile Asp Val Ser
            740             745             750

Lys Pro Val Lys Leu Ser Glu Gly Leu Asp Glu Val Lys Ala Asp Tyr
            755             760             765

Ser Leu Ala Val Lys Met Leu Lys Glu Ile Arg Asp Ala Tyr Arg Asn
    770             775             780

Ile Gly His Lys Leu Lys Glu Gly Glu Thr Tyr Ile Ser Arg Asp Leu
785             790             795                 800

Gln Phe Ile Ser Lys Val Ile Gln Ser Glu Gly Val Met His Pro Thr
            805             810             815

Pro Ile Lys Lys Ile Leu Arg Val Gly Pro Trp Ile Asn Thr Ile Leu
            820             825             830

Asp Asp Ile Lys Thr Ser Ala Glu Ser Ile Gly Ser Leu Cys Gln Glu
            835             840             845

Leu Glu Phe Arg Gly Glu Ser Ile Ile Val Ser Leu Ile Leu Arg Asn
    850             855             860

Phe Trp Leu Tyr Asn Leu Tyr Met His Glu Ser Lys Gln His Pro Leu
865             870             875                 880
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Ala Gly Lys Gln Leu Phe Lys Gln Leu Asn Lys Thr Leu Thr Ser Val
                885                     890                 895


Gln Arg Phe Phe Glu Ile Lys Lys Glu Asn Glu Val Val Asp Leu Trp
            900                     905                 910


Met Asn Ile Pro Met Gln Phe Gly Gly Gly Asp Pro Val Val Phe Tyr
            915                     920                 925


Arg Ser Phe Tyr Arg Arg Thr Pro Asp Phe Leu Thr Glu Ala Ile Ser
    930                     935                 940


His Val Asp Ile Leu Leu Arg Ile Ser Ala Asn Ile Arg Asn Glu Ala
945                     950                 955                 960


Lys Ile Ser Phe Phe Lys Ala Leu Leu Ser Ile Glu Lys Asn Glu Arg
                965                     970                 975


Ala Thr Leu Thr Thr Leu Met Arg Asp Pro Gln Ala Val Gly Ser Glu
                980                     985                 990


Arg Gln Ala Lys Val Thr Ser Asp  Ile Asn Arg Thr Ala  Val Thr Ser
        995                 1000                 1005


Ile Leu  Ser Leu Ser Pro Asn  Gln Leu Phe Ser Asp  Ser Ala Ile
    1010                 1015                 1020


His Tyr  Ser Arg Asn Glu Glu  Glu Val Gly Ile Ile  Ala Asp Asn
    1025                 1030                 1035


Ile Thr  Pro Val Tyr Pro His  Gly Leu Arg Val Leu  Tyr Glu Ser
    1040                 1045                 1050


Leu Pro  Phe His Lys Ala Glu  Lys Val Val Asn Met  Ile Ser Gly
    1055                 1060                 1065


Thr Lys  Ser Ile Thr Asn Leu  Leu Gln Arg Thr Ser  Ala Ile Asn
    1070                 1075                 1080


Gly Glu  Asp Ile Asp Arg Ala  Val Ser Met Met Leu  Glu Asn Leu
    1085                 1090                 1095


Gly Leu  Leu Ser Arg Ile Leu  Ser Val Val Val Asp  Ser Ile Glu
    1100                 1105                 1110
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44

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Ile Pro  Thr Lys Ser Asn Gly  Arg Leu Ile Cys Cys  Gln Ile Ser
    1115                1120               1125


Arg Thr  Leu Arg Glu Thr Ser  Trp Asn Asn Met Glu  Ile Val Gly
    1130                1135               1140


Val Thr  Ser Pro Ser Ile Thr  Thr Cys Met Asp Val  Ile Tyr Ala
    1145                1150               1155


Thr Ser  Ser His Leu Lys Gly  Ile Ile Ile Glu Lys  Phe Ser Thr
    1160                1165               1170


Asp Arg  Thr Thr Arg Gly Gln  Arg Gly Pro Lys Ser  Pro Trp Val
    1175                1180               1185


Gly Ser  Ser Thr Gln Glu Lys  Lys Leu Val Pro Val  Tyr Asn Arg
    1190                1195               1200


Gln Ile  Leu Ser Lys Gln Gln  Arg Glu Gln Leu Glu  Ala Ile Gly
    1205                1210               1215


Lys Met  Arg Trp Val Tyr Lys  Gly Thr Pro Gly Leu  Arg Arg Leu
    1220                1225               1230


Leu Asn  Lys Ile Cys Leu Gly  Ser Leu Gly Ile Ser  Tyr Lys Cys
    1235                1240               1245


Val Lys  Pro Leu Leu Pro Arg  Phe Met Ser Val Asn  Phe Leu His
    1250                1255               1260


Arg Leu  Ser Val Ser Ser Arg  Pro Met Glu Phe Pro  Ala Ser Val
    1265                1270               1275


Pro Ala  Tyr Arg Thr Thr Asn  Tyr His Phe Asp Thr  Ser Pro Ile
    1280                1285               1290


Asn Gln  Ala Leu Ser Glu Arg  Phe Gly Asn Glu Asp  Ile Asn Leu
    1295                1300               1305


Val Phe  Gln Asn Ala Ile Ser  Cys Gly Ile Ser Ile  Met Ser Val
    1310                1315               1320


Val Glu  Gln Leu Thr Gly Arg  Ser Pro Lys Gln Leu  Val Leu Ile
    1325                1330               1335
```

45

Pro Gln   Leu Glu Glu Ile Asp   Ile Met Pro Pro Pro   Val Phe Gln
    1340   1345   1350

Gly Lys   Phe Asn Tyr Lys Leu   Val Asp Lys Ile Thr   Ser Asp Gln
    1355   1360   1365

His Ile   Phe Ser Pro Asp Lys   Ile Asp Met Leu Thr   Leu Gly Lys
    1370   1375   1380

Met Leu   Met Pro Thr Ile Lys   Gly Gln Lys Thr Asp   Gln Phe Leu
    1385   1390   1395

Asn Lys   Arg Glu Asn Tyr Phe   His Gly Asn Asn Leu   Ile Glu Ser
    1400   1405   1410

Leu Ser   Ala Ala Leu Ala Cys   His Trp Cys Gly Ile   Leu Thr Glu
    1415   1420   1425

Gln Cys   Ile Glu Asn Asn Ile   Phe Lys Lys Asp Trp   Gly Asp Gly
    1430   1435   1440

Phe Ile   Ser Asp His Ala Phe   Met Asp Phe Lys Ile   Phe Leu Cys
    1445   1450   1455

Val Phe   Lys Thr Lys Leu Leu   Cys Ser Trp Gly Ser   Gln Gly Lys
    1460   1465   1470

Asn Ile   Lys Asp Glu Asp Ile   Val Asp Glu Ser Ile   Asp Lys Leu
    1475   1480   1485

Leu Arg   Ile Asp Asn Thr Phe   Trp Arg Met Phe Ser   Lys Val Met
    1490   1495   1500

Phe Glu   Ser Lys Val Lys Lys   Arg Ile Met Leu Tyr   Asp Val Lys
    1505   1510   1515

Phe Leu   Ser Leu Val Gly Tyr   Ile Gly Phe Lys Asn   Trp Phe Ile
    1520   1525   1530

Glu Gln   Leu Arg Ser Ala Glu   Leu His Glu Val Pro   Trp Ile Val
    1535   1540   1545

Asn Ala   Glu Gly Asp Leu Val   Glu Ile Lys Ser Ile   Lys Ile Tyr
    1550   1555   1560

Leu Gln Leu Ile Glu Gln Ser Leu Phe Leu Arg Ile Thr Val Leu
    1565   1570   1575

Asn Tyr Thr Asp Met Ala His Ala Leu Thr Arg Leu Ile Arg Lys
    1580   1585   1590

Lys Leu Met Cys Asp Asn Ala Leu Leu Thr Pro Ile Pro Ser Pro
    1595   1600   1605

Met Val Asn Leu Thr Gln Val Ile Asp Pro Thr Glu Gln Leu Ala
    1610   1615   1620

Tyr Phe Pro Lys Ile Thr Phe Glu Arg Leu Lys Asn Tyr Asp Thr
    1625   1630   1635

Ser Ser Asn Tyr Ala Lys Gly Lys Leu Thr Arg Asn Tyr Met Ile
    1640   1645   1650

Leu Leu Pro Trp Gln His Val Asn Arg Tyr Asn Phe Val Phe Ser
    1655   1660   1665

Ser Thr Gly Cys Lys Val Ser Leu Lys Thr Cys Ile Gly Lys Leu
    1670   1675   1680

Met Lys Asp Leu Asn Pro Lys Val Leu Tyr Phe Ile Gly Glu Gly
    1685   1690   1695

Ala Gly Asn Trp Met Ala Arg Thr Ala Cys Glu Tyr Pro Asp Ile
    1700   1705   1710

Lys Phe Val Tyr Arg Ser Leu Lys Asp Asp Leu Asp His His Tyr
    1715   1720   1725

Pro Leu Glu Tyr Gln Arg Val Ile Gly Glu Leu Ser Arg Ile Ile
    1730   1735   1740

Asp Ser Gly Glu Gly Leu Ser Met Glu Thr Thr Asp Ala Thr Gln
    1745   1750   1755

Lys Thr His Trp Asp Leu Ile His Arg Val Ser Lys Asp Ala Leu
    1760   1765   1770

Leu Ile Thr Leu Cys Asp Ala Glu Phe Lys Asp Arg Asp Asp Phe
    1775   1780   1785

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```
Phe Lys   Met Val Ile Leu Trp   Arg Lys His Val Leu   Ser Cys Arg
    1790              1795               1800

Ile Cys   Thr Thr Tyr Gly Thr   Asp Leu Tyr Leu Phe   Ala Lys Tyr
    1805              1810               1815

His Ala   Lys Asp Cys Asn Val   Lys Leu Pro Phe Phe   Val Arg Ser
    1820              1825               1830

Val Ala   Thr Phe Ile Met Gln   Gly Ser Lys Leu Ser   Gly Ser Glu
    1835              1840               1845

Cys Tyr   Ile Leu Leu Thr Leu   Gly His His Asn Asn   Leu Pro Cys
    1850              1855               1860

His Gly   Glu Ile Gln Asn Ser   Lys Met Lys Ile Ala   Val Cys Asn
    1865              1870               1875

Asp Phe   Tyr Ala Ala Lys Lys   Leu Asp Asn Lys Ser   Ile Glu Ala
    1880              1885               1890

Asn Cys   Lys Ser Leu Leu Ser   Gly Leu Arg Ile Pro   Ile Asn Lys
    1895              1900               1905

Lys Glu   Leu Asn Arg Gln Arg   Arg Leu Leu Thr Leu   Gln Ser Asn
    1910              1915               1920

His Ser   Ser Val Ala Thr Val   Gly Gly Ser Lys Val   Ile Glu Ser
    1925              1930               1935

Lys Trp   Leu Thr Asn Lys Ala   Asn Thr Ile Ile Asp   Trp Leu Glu
    1940              1945               1950

His Ile   Leu Asn Ser Pro Lys   Gly Glu Leu Asn Tyr   Asp Phe Phe
    1955              1960               1965

Glu Ala   Leu Glu Asn Thr Tyr   Pro Asn Met Ile Lys   Leu Ile Asp
    1970              1975               1980

Asn Leu   Gly Asn Ala Glu Ile   Lys Lys Leu Ile Lys   Val Thr Gly
    1985              1990               1995

Tyr Met   Leu Val Ser Lys Lys
    2000              2005
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48

<210> 13
<211> 2165
<212> PRT
<213> human respiratory syncytial virus strain A2

<400> 13

Met Asp Pro Ile Ile Asn Gly Asn Ser Ala Asn Val Tyr Leu Thr Asp
1   5   10   15

Ser Tyr Leu Lys Gly Val Ile Ser Phe Ser Glu Cys Asn Ala Leu Gly
            20   25   30

Ser Tyr Ile Phe Asn Gly Pro Tyr Leu Lys Asn Asp Tyr Thr Asn Leu
        35   40   45

Ile Ser Arg Gln Asn Pro Leu Ile Glu His Met Asn Leu Lys Lys Leu
    50   55   60

Asn Ile Thr Gln Ser Leu Ile Ser Lys Tyr His Lys Gly Glu Ile Lys
65   70   75   80

Leu Glu Glu Pro Thr Tyr Phe Gln Ser Leu Leu Met Thr Tyr Lys Ser
                85   90   95

Met Thr Ser Ser Glu Gln Ile Ala Thr Thr Asn Leu Leu Lys Lys Ile
            100   105   110

Ile Arg Arg Ala Ile Glu Ile Ser Asp Val Lys Val Tyr Ala Ile Leu
        115   120   125

Asn Lys Leu Gly Leu Lys Glu Lys Asp Lys Ile Lys Ser Asn Asn Gly
    130   135   140

Gln Asp Glu Asp Asn Ser Val Ile Thr Thr Ile Ile Lys Asp Asp Ile
145   150   155   160

Leu Ser Ala Val Lys Asp Asn Gln Ser His Leu Lys Ala Asp Lys Asn
                165   170   175

His Ser Thr Lys Gln Lys Asp Thr Ile Lys Thr Thr Leu Leu Lys Lys
            180   185   190

Leu Met Cys Ser Met Gln His Pro Pro Ser Trp Leu Ile His Trp Phe
            195   200   205

Asn Leu Tyr Thr Lys Leu Asn Asn Ile Leu Thr Gln Tyr Arg Ser Asn
                                    49

                210   215   220

Glu Val Lys Asn His Gly Phe Thr Leu Ile Asp Asn Gln Thr Leu Ser
225   230   235   240

Gly Phe Gln Phe Ile Leu Asn Gln Tyr Gly Cys Ile Val Tyr His Lys
                    245   250   255

Glu Leu Lys Arg Ile Thr Val Thr Thr Tyr Asn Gln Phe Leu Thr Trp
                260   265   270

Lys Asp Ile Ser Leu Ser Arg Leu Asn Val Cys Leu Ile Thr Trp Ile
            275   280   285

Ser Asn Cys Leu Asn Thr Leu Asn Lys Ser Leu Gly Leu Arg Cys Gly
    290   295   300

Phe Asn Asn Val Ile Leu Thr Gln Leu Phe Leu Tyr Gly Asp Cys Ile
305   310   315   320

Leu Lys Leu Phe His Asn Glu Gly Phe Tyr Ile Ile Lys Glu Val Glu
                325   330   335

Gly Phe Ile Met Ser Leu Ile Leu Asn Ile Thr Glu Glu Asp Gln Phe
                340   345   350

Arg Lys Arg Phe Tyr Asn Ser Met Leu Asn Asn Ile Thr Asp Ala Ala
            355   360   365

Asn Lys Ala Gln Lys Asn Leu Leu Ser Arg Val Cys His Thr Leu Leu
    370   375   380

Asp Lys Thr Val Ser Asp Asn Ile Ile Asn Gly Arg Trp Ile Ile Leu
385   390   395   400

Leu Ser Lys Phe Leu Lys Leu Ile Lys Leu Ala Gly Asp Asn Asn Leu
                405   410   415

Asn Asn Leu Ser Glu Leu Tyr Phe Leu Phe Arg Ile Phe Gly His Pro
                420   425   430

Met Val Asp Glu Arg Gln Ala Met Asp Ala Val Lys Ile Asn Cys Asn
            435   440   445

Glu Thr Lys Phe Tyr Leu Leu Ser Ser Leu Ser Met Leu Arg Gly Ala
                              50

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              450                    455                    460


Phe Ile Tyr Arg Ile Ile Lys Gly Phe Val Asn Asn Tyr Asn Arg Trp
465             470             475             480


Pro Thr Leu Arg Asn Ala Ile Val Leu Pro Leu Arg Trp Leu Thr Tyr
            485             490             495


Tyr Lys Leu Asn Thr Tyr Pro Ser Leu Leu Glu Leu Thr Glu Arg Asp
            500             505             510


Leu Ile Val Leu Ser Gly Leu Arg Phe Tyr Arg Glu Phe Arg Leu Pro
            515             520             525


Lys Lys Val Asp Leu Glu Met Ile Ile Asn Asp Lys Ala Ile Ser Pro
    530             535             540


Pro Lys Asn Leu Ile Trp Thr Ser Phe Pro Arg Asn Tyr Met Pro Ser
545             550             555             560


His Ile Gln Asn Tyr Ile Glu His Glu Lys Leu Lys Phe Ser Glu Ser
            565             570             575


Asp Lys Ser Arg Arg Val Leu Glu Tyr Tyr Leu Arg Asp Asn Lys Phe
            580             585             590


Asn Glu Cys Asp Leu Tyr Asn Cys Val Val Asn Gln Ser Tyr Leu Asn
            595             600             605


Asn Pro Asn His Val Val Ser Leu Thr Gly Lys Glu Arg Glu Leu Ser
    610             615             620


Val Gly Arg Met Phe Ala Met Gln Pro Gly Met Phe Arg Gln Val Gln
625             630             635             640


Ile Leu Ala Glu Lys Met Ile Ala Glu Asn Ile Leu Gln Phe Phe Pro
            645             650             655


Glu Ser Leu Thr Arg Tyr Gly Asp Leu Glu Leu Gln Lys Ile Leu Glu
            660             665             670


Leu Lys Ala Gly Ile Ser Asn Lys Ser Asn Arg Tyr Asn Asp Asn Tyr
            675             680             685


Asn Asn Tyr Ile Ser Lys Cys Ser Ile Ile Thr Asp Leu Ser Lys Phe
                            51
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          690                      695                      700
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Asn Gln Ala Phe Arg Tyr Glu Thr Ser Cys Ile Cys Ser Asp Val Leu
705   710   715   720

Asp Glu Leu His Gly Val Gln Ser Leu Phe Ser Trp Leu His Leu Thr
            725   730   735

Ile Pro His Val Thr Ile Ile Cys Thr Tyr Arg His Ala Pro Pro Tyr
            740   745   750

Ile Gly Asp His Ile Val Asp Leu Asn Asn Val Asp Glu Gln Ser Gly
            755   760   765

Leu Tyr Arg Tyr His Met Gly Gly Ile Glu Gly Trp Cys Gln Lys Leu
    770   775   780

Trp Thr Ile Glu Ala Ile Ser Leu Leu Asp Leu Ile Ser Leu Lys Gly
785   790   795   800

Lys Phe Ser Ile Thr Ala Leu Ile Asn Gly Asp Asn Gln Ser Ile Asp
            805   810   815

Ile Ser Lys Pro Ile Arg Leu Met Glu Gly Gln Thr His Ala Gln Ala
            820   825   830

Asp Tyr Leu Leu Ala Leu Asn Ser Leu Lys Leu Leu Tyr Lys Glu Tyr
        835   840   845

Ala Gly Ile Gly His Lys Leu Lys Gly Thr Glu Thr Tyr Ile Ser Arg
    850   855   860

Asp Met Gln Phe Met Ser Lys Thr Ile Gln His Asn Gly Val Tyr Tyr
865   870   875   880

Pro Ala Ser Ile Lys Lys Val Leu Arg Val Gly Pro Trp Ile Asn Thr
            885   890   895

Ile Leu Asp Asp Phe Lys Val Ser Leu Glu Ser Ile Gly Ser Leu Thr
            900   905   910

Gln Glu Leu Glu Tyr Arg Gly Glu Ser Leu Leu Cys Ser Leu Ile Phe
        915   920   925

Arg Asn Val Trp Leu Tyr Asn Gln Ile Ala Leu Gln Leu Lys Asn His

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        930                       935                          940


Ala Leu Cys Asn Asn Lys Leu Tyr Leu Asp Ile Leu Lys Val Leu Lys
945                  950              955                  960


His Leu Lys Thr Phe Phe Asn Leu Asp Asn Ile Asp Thr Ala Leu Thr
                965              970                  975


Leu Tyr Met Asn Leu Pro Met Leu Phe Gly Gly Gly Asp Pro Asn Leu
            980              985                  990


Leu Tyr Arg Ser Phe Tyr Arg Arg  Thr Pro Asp Phe Leu  Thr Glu Ala
        995              1000              1005


Ile Val  His Ser Val Phe Ile  Leu Ser Tyr Tyr Thr  Asn His Asp
    1010              1015              1020


Leu Lys  Asp Lys Leu Gln Asp  Leu Ser Asp Asp Arg  Leu Asn Lys
    1025              1030              1035


Phe Leu  Thr Cys Ile Ile Thr  Phe Asp Lys Asn Pro  Asn Ala Glu
    1040              1045              1050


Phe Val  Thr Leu Met Arg Asp  Pro Gln Ala Leu Gly  Ser Glu Arg
    1055              1060              1065


Gln Ala  Lys Ile Thr Ser Glu  Ile Asn Arg Leu Ala  Val Thr Glu
    1070              1075              1080


Val Leu  Ser Thr Ala Pro Asn  Lys Ile Phe Ser Lys  Ser Ala Gln
    1085              1090              1095


His Tyr  Thr Thr Thr Glu Ile  Asp Leu Asn Asp Ile  Met Gln Asn
    1100              1105              1110


Ile Glu  Pro Thr Tyr Pro His  Gly Leu Arg Val Val  Tyr Glu Ser
    1115              1120              1125


Leu Pro  Phe Tyr Lys Ala Glu  Lys Ile Val Asn Leu  Ile Ser Gly
    1130              1135              1140


Thr Lys  Ser Ile Thr Asn Ile  Leu Glu Lys Thr Ser  Ala Ile Asp
    1145              1150              1155


Leu Thr  Asp Ile Asp Arg Ala  Thr Glu Met Met Arg  Lys Asn Ile
                                53
```

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        1160                      1165                      1170
```

Thr Leu Leu Ile Arg Ile Leu Pro Leu Asp Cys Asn Arg Asp Lys
    1175   1180   1185

Arg Glu Ile Leu Ser Met Glu Asn Leu Ser Ile Thr Glu Leu Ser
    1190   1195   1200

Lys Tyr Val Arg Glu Arg Ser Trp Ser Leu Ser Asn Ile Val Gly
    1205   1210   1215

Val Thr Ser Pro Ser Ile Met Tyr Thr Met Asp Ile Lys Tyr Thr
    1220   1225   1230

Thr Ser Thr Ile Ser Ser Gly Ile Ile Ile Glu Lys Tyr Asn Val
    1235   1240   1245

Asn Ser Leu Thr Arg Gly Glu Arg Gly Pro Thr Lys Pro Trp Val
    1250   1255   1260

Gly Ser Ser Thr Gln Glu Lys Lys Thr Met Pro Val Tyr Asn Arg
    1265   1270   1275

Gln Val Leu Thr Lys Lys Gln Arg Asp Gln Ile Asp Leu Leu Ala
    1280   1285   1290

Lys Leu Asp Trp Val Tyr Ala Ser Ile Asp Asn Lys Asp Glu Phe
    1295   1300   1305

Met Glu Glu Leu Ser Ile Gly Thr Leu Gly Leu Thr Tyr Glu Lys
    1310   1315   1320

Ala Lys Lys Leu Phe Pro Gln Tyr Leu Ser Val Asn Tyr Leu His
    1325   1330   1335

Arg Leu Thr Val Ser Ser Arg Pro Cys Glu Phe Pro Ala Ser Ile
    1340   1345   1350

Pro Ala Tyr Arg Thr Thr Asn Tyr His Phe Asp Thr Ser Pro Ile
    1355   1360   1365

Asn Arg Ile Leu Thr Glu Lys Tyr Gly Asp Glu Asp Ile Asp Ile
    1370   1375   1380

Val Phe Gln Asn Cys Ile Ser Phe Gly Leu Ser Leu Met Ser Val

                                54

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          1385                    1390                    1395

   Val Glu Gln Phe Thr Asn Val Cys Pro Asn Arg Ile Ile Leu Ile
       1400                    1405                    1410


   Pro Lys Leu Asn Glu Ile His Leu Met Lys Pro Pro Ile Phe Thr
       1415                    1420                    1425


   Gly Asp Val Asp Ile His Lys Leu Lys Gln Val Ile Gln Lys Gln
       1430                    1435                    1440


   His Met Phe Leu Pro Asp Lys Ile Ser Leu Thr Gln Tyr Val Glu
       1445                    1450                    1455


   Leu Phe Leu Ser Asn Lys Thr Leu Lys Ser Gly Ser His Val Asn
       1460                    1465                    1470


   Ser Asn Leu Ile Leu Ala His Lys Ile Ser Asp Tyr Phe His Asn
       1475                    1480                    1485


   Thr Tyr Ile Leu Ser Thr Asn Leu Ala Gly His Trp Ile Leu Ile
       1490                    1495                    1500


   Ile Gln Leu Met Lys Asp Ser Lys Gly Ile Phe Glu Lys Asp Trp
       1505                    1510                    1515


   Gly Glu Gly Tyr Ile Thr Asp His Met Phe Ile Asn Leu Lys Val
       1520                    1525                    1530


   Phe Phe Asn Ala Tyr Lys Thr Tyr Leu Leu Cys Phe His Lys Gly
       1535                    1540                    1545


   Tyr Gly Lys Ala Lys Leu Glu Cys Asp Met Asn Thr Ser Asp Leu
       1550                    1555                    1560


   Leu Cys Val Leu Glu Leu Ile Asp Ser Ser Tyr Trp Lys Ser Met
       1565                    1570                    1575


   Ser Lys Val Phe Leu Glu Gln Lys Val Ile Lys Tyr Ile Leu Ser
       1580                    1585                    1590


   Gln Asp Ala Ser Leu His Arg Val Lys Gly Cys His Ser Phe Lys
       1595                    1600                    1605


   Leu Trp Phe Leu Lys Arg Leu Asn Val Ala Glu Phe Thr Val Cys
                            55
```

```
            1610                    1615                    1620


Pro Trp Val Val Asn Ile Asp   Tyr His Pro Thr His   Met Lys Ala
        1625                    1630                    1635


Ile Leu Thr Tyr Ile Asp Leu   Val Arg Met Gly Leu   Ile Asn Ile
        1640                    1645                    1650


Asp Arg Ile His Ile Lys Asn   Lys His Lys Phe Asn   Asp Glu Phe
        1655                    1660                    1665


Tyr Thr Ser Asn Leu Phe Tyr   Ile Asn Tyr Asn Phe   Ser Asp Asn
        1670                    1675                    1680


Thr His Leu Leu Thr Lys His   Ile Arg Ile Ala Asn   Ser Glu Leu
        1685                    1690                    1695


Glu Asn Asn Tyr Asn Lys Leu   Tyr His Pro Thr Pro   Glu Thr Leu
        1700                    1705                    1710


Glu Asn Ile Leu Ala Asn Pro   Ile Lys Ser Asn Asp   Lys Lys Thr
        1715                    1720                    1725


Leu Asn Asp Tyr Cys Ile Gly   Lys Asn Val Asp Ser   Ile Met Leu
        1730                    1735                    1740


Pro Leu Leu Ser Asn Lys Lys   Leu Ile Lys Ser Ser   Ala Met Ile
        1745                    1750                    1755


Arg Thr Asn Tyr Ser Lys Gln   Asp Leu Tyr Asn Leu   Phe Pro Met
        1760                    1765                    1770


Val Val Ile Asp Arg Ile Ile   Asp His Ser Gly Asn   Thr Ala Lys
        1775                    1780                    1785


Ser Asn Gln Leu Tyr Thr Thr   Thr Ser His Gln Ile   Ser Leu Val
        1790                    1795                    1800


His Asn Ser Thr Ser Leu Tyr   Cys Met Leu Pro Trp   His His Ile
        1805                    1810                    1815


Asn Arg Phe Asn Phe Val Phe   Ser Ser Thr Gly Cys   Lys Ile Ser
        1820                    1825                    1830


Ile Glu Tyr Ile Leu Lys Asp   Leu Lys Ile Lys Asp   Pro Asn Cys
```

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```
        1835                      1840                      1845


   Ile Ala  Phe Ile Gly Glu Gly  Ala Gly Asn Leu Leu  Leu Arg Thr
        1850                      1855                      1860


   Val Val  Glu Leu His Pro Asp  Ile Arg Tyr Ile Tyr  Arg Ser Leu
        1865                      1870                      1875


   Lys Asp  Cys Asn Asp His Ser  Leu Pro Ile Glu Phe  Leu Arg Leu
        1880                      1885                      1890


   Tyr Asn  Gly His Ile Asn Ile  Asp Tyr Gly Glu Asn  Leu Thr Ile
        1895                      1900                      1905


   Pro Ala  Thr Asp Ala Thr Asn  Asn Ile His Trp Ser  Tyr Leu His
        1910                      1915                      1920


   Ile Lys  Phe Ala Glu Pro Ile  Ser Leu Phe Val Cys  Asp Ala Glu
        1925                      1930                      1935


   Leu Ser  Val Thr Val Asn Trp  Ser Lys Ile Ile Ile  Glu Trp Ser
        1940                      1945                      1950


   Lys His  Val Arg Lys Cys Lys  Tyr Cys Ser Ser Val  Asn Lys Cys
        1955                      1960                      1965


   Met Leu  Ile Val Lys Tyr His  Ala Gln Asp Asp Ile  Asp Phe Lys
        1970                      1975                      1980


   Leu Asp  Asn Ile Thr Ile Leu  Lys Thr Tyr Val Cys  Leu Gly Ser
        1985                      1990                      1995


   Lys Leu  Lys Gly Ser Glu Val  Tyr Leu Val Leu Thr  Ile Gly Pro
        2000                      2005                      2010


   Ala Asn  Ile Phe Pro Val Phe  Asn Val Val Gln Asn  Ala Lys Leu
        2015                      2020                      2025


   Ile Leu  Ser Arg Thr Lys Asn  Phe Ile Met Pro Lys  Lys Ala Asp
        2030                      2035                      2040


   Lys Glu  Ser Ile Asp Ala Asn  Ile Lys Ser Leu Ile  Pro Phe Leu
        2045                      2050                      2055


   Cys Tyr  Pro Ile Thr Lys Lys  Gly Ile Asn Thr Ala  Leu Ser Lys
```

```
         2060                      2065                      2070
```

```
Leu Lys  Ser Val Val Ser Gly  Asp Ile Leu Ser Tyr  Ser Ile Ala
        2075                2080                2085
```

```
Gly Arg  Asn Glu Val Phe Ser  Asn Lys Leu Ile Asn  His Lys His
        2090                2095                2100
```

```
Met Asn  Ile Leu Lys Trp Phe  Asn His Val Leu Asn  Phe Arg Ser
        2105                2110                2115
```

```
Thr Glu · Leu Asn Tyr Asn His  Leu Tyr Met Val Glu  Ser Thr Tyr
        2120                2125                2130
```

```
Pro Tyr  Leu Ser Glu Leu Leu  Asn Ser Leu Thr Thr  Asn Glu Leu
        2135                2140                2145
```

```
Lys Lys  Leu Ile Lys Ile Thr  Gly Ser Leu Leu Tyr  Asn Phe His
        2150                2155                2160
```

```
Asn Glu
        2165
```

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<210>   14
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<213>   human parainfluenza virus 3

<400>   14
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```
Met Asp Thr Glu Ser Asn Asn Gly Thr Val Ser Asp Ile Leu Tyr Pro
1                   5                   10                  15
```

```
Glu Cys His Leu Asn Ser Pro Ile Val Lys Gly Lys Ile Ala Gln Leu
                20                  25                  30
```

```
His Thr Ile Met Ser Leu Pro Gln Pro Tyr Asp Met Asp Asp Asp Ser
            35                  40                  45
```

```
Ile Leu Val Ile Thr Arg Gln Lys Ile Lys Leu Asn Lys Leu Asp Lys
    50                  55                  60
```

```
Arg Gln Arg Ser Ile Arg Arg Leu Lys Leu Ile Leu Thr Glu Lys Val
65                  70                  75                  80
```

```
Asn Asp Leu Gly Lys Tyr Thr Phe Ile Arg Tyr Pro Glu Met Ser Lys
                85                  90                  95
```

```
Glu Met Phe Lys Leu His Ile Pro Gly Ile Asn Ser Lys Val Thr Glu
        100             105             110

Leu Leu Leu Lys Ala Asp Arg Thr Tyr Ser Gln Met Thr Asp Gly Leu
        115             120             125

Arg Asp Leu Trp Ile Asn Val Leu Ser Lys Leu Ala Ser Lys Asn Asp
    130             135             140

Gly Ser Asn Tyr Asp Leu Asn Glu Glu Ile Asn Asn Ile Ser Lys Val
145             150             155             160

His Thr Thr Tyr Lys Ser Asp Lys Trp Tyr Asn Pro Phe Lys Thr Trp
            165             170             175

Phe Thr Ile Lys Tyr Asp Met Arg Arg Leu Gln Lys Ala Arg Asn Glu
            180             185             190

Val Thr Phe Asn Met Gly Lys Asp Tyr Asn Leu Leu Glu Asp Gln Lys
        195             200             205

Asn Phe Leu Leu Ile His Pro Glu Leu Val Leu Ile Leu Asp Lys Gln
    210             215             220

Asn Tyr Asn Gly Tyr Leu Ile Thr Pro Glu Leu Val Leu Pro Tyr Cys
225             230             235             240

Asp Val Val Glu Gly Arg Trp Asn Ile Ser Ala Cys Ala Lys Leu Asp
            245             250             255

Pro Lys Leu Gln Ser Met Tyr Gln Lys Gly Asn Asn Leu Trp Glu Val
        260             265             270

Ile Asp Lys Leu Phe Pro Ile Met Gly Glu Lys Thr Phe Asp Val Ile
        275             280             285

Ser Leu Leu Glu Pro Leu Ala Leu Ser Leu Ile Gln Thr His Asp Pro
    290             295             300

Val Lys Gln Leu Arg Gly Ala Phe Leu Asn His Val Leu Ser Glu Met
305             310             315             320

Glu Leu Ile Phe Glu Ser Arg Glu Ser Ile Lys Glu Phe Leu Ser Val
            325             330             335
```

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```
Asp Tyr Ile Asp Lys Ile Leu Asp Ile Phe Asn Lys Ser Thr Ile Asp
        340             345                 350

Glu Ile Ala Glu Ile Phe Ser Phe Phe Arg Thr Phe Gly His Pro Pro
        355             360                 365

Leu Glu Ala Ser Ile Ala Ala Glu Lys Val Arg Lys Tyr Met Tyr Ile
    370             375                 380

Gly Lys Gln Leu Lys Phe Asp Thr Ile Asn Lys Cys His Ala Ile Phe
385             390                 395                 400

Cys Thr Ile Ile Ile Asn Gly Tyr Arg Glu Arg His Gly Gly Gln Trp
            405             410                 415

Pro Pro Val Thr Leu Pro Asp His Ala His Glu Phe Ile Ile Asn Ala
            420             425                 430

Tyr Gly Ser Asn Ser Ala Ile Ser Tyr Glu Asn Ala Val Asp Tyr Tyr
        435             440                 445

Gln Ser Phe Ile Gly Ile Lys Phe Asn Lys Phe Ile Glu Pro Gln Leu
        450             455                 460

Asp Glu Asp Leu Thr Ile Tyr Met Lys Asp Lys Ala Leu Ser Pro Lys
465             470                 475                 480

Lys Ser Asn Trp Asp Thr Val Ser Pro Ala Ser Asn Leu Leu Tyr Arg
            485             490                 495

Thr Asn Ala Ser Asn Glu Ser Arg Arg Leu Val Glu Lys Phe Ile Ala
            500             505                 510

Asp Ser Lys Phe Asp Pro Asn Gln Ile Leu Asp Tyr Val Glu Ser Gly
        515             520                 525

Asp Trp Leu Asp Asp Pro Glu Phe Asn Ile Ser Tyr Ser Leu Lys Glu
    530             535                 540

Lys Glu Ile Lys Gln Glu Gly Arg Leu Phe Ala Lys Met Thr Tyr Lys
545             550                 555                 560

Met Arg Ala Thr Gln Val Leu Ser Glu Thr Leu Leu Ala Asn Asn Ile
            565             570                 575
```

60

```
Gly Lys Phe Phe Gln Glu Asn Gly Met Val Lys Gly Glu Ile Glu Leu
        580                 585                 590

Leu Lys Arg Leu Thr Thr Ile Ser Ile Ser Gly Val Pro Arg Tyr Asn
        595                 600                 605

Glu Val Tyr Asn Asn Ser Lys Ser His Thr Asp Asp Leu Lys Thr Tyr
    610                 615                 620

Asn Lys Ile Ser Asn Leu Asn Leu Ser Ser Asn Gln Lys Ser Lys Lys
625                 630                 635                 640

Phe Glu Phe Lys Ser Thr Asp Ile Tyr Asn Asp Gly Tyr Glu Thr Val
            645                 650                 655

Ser Cys Phe Leu Thr Thr Asp Leu Lys Lys Tyr Cys Leu Asn Trp Arg
            660                 665                 670

Tyr Glu Ser Thr Ala Leu Phe Gly Glu Thr Cys Asn Gln Ile Phe Gly
        675                 680                 685

Leu Asn Lys Leu Phe Asn Trp Leu His Pro Arg Leu Glu Gly Ser Thr
    690                 695                 700

Ile Tyr Val Gly Asp Pro Tyr Cys Pro Pro Ser Asp Lys Glu His Ile
705                 710                 715                 720

Ser Leu Glu Asp His Pro Asp Ser Gly Phe Tyr Val His Asn Pro Arg
            725                 730                 735

Gly Gly Ile Glu Gly Phe Cys Gln Lys Leu Trp Thr Leu Ile Ser Ile
        740                 745                 750

Ser Ala Ile His Leu Ala Ala Val Arg Ile Gly Val Arg Val Thr Ala
        755                 760                 765

Met Val Gln Gly Asp Asn Gln Ala Ile Ala Val Thr Thr Arg Val Pro
    770                 775                 780

Asn Asn Tyr Asp Tyr Arg Val Lys Lys Glu Ile Val Tyr Lys Asp Val
785                 790                 795                 800

Val Arg Phe Phe Asp Ser Leu Arg Glu Val Met Asp Asp Leu Gly His
            805                 810                 815
```

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Glu Leu Lys Leu Asn Glu Thr Ile Ile Ser Ser Lys Met Phe Ile Tyr
        820   825   830

Ser Lys Arg Ile Tyr Tyr Asp Gly Arg Ile Leu Pro Gln Ala Leu Lys
        835   840   845

Ala Leu Ser Arg Cys Val Phe Trp Ser Glu Thr Val Ile Asp Glu Thr
    850   855   860

Arg Ser Ala Ser Ser Asn Leu Ala Thr Ser Phe Ala Lys Ala Ile Glu
865   870   875   880

Asn Gly Tyr Ser Pro Val Leu Gly Tyr Ala Cys Ser Ile Phe Lys Asn
            885   890   895

Ile Gln Gln Leu Tyr Ile Ala Leu Gly Met Asn Ile Asn Pro Thr Ile
            900   905   910

Thr Gln Asn Ile Lys Asp Leu Tyr Phe Arg Asn Pro Asn Trp Met Gln
            915   920   925

Tyr Ala Ser Leu Ile Pro Ala Ser Val Gly Gly Phe Asn Tyr Met Ala
    930   935   940

Met Ser Arg Cys Phe Val Arg Asn Ile Gly Asp Pro Ser Val Ala Ala
945   950   955   960

Leu Ala Asp Ile Lys Arg Phe Ile Lys Ala Asn Leu Leu Asp Arg Ser
            965   970   975

Val Leu Tyr Arg Ile Met Asn Gln Glu Pro Gly Glu Ser Ser Phe Leu
            980   985   990

Asp Trp Ala Ser Asp Pro Tyr Ser Cys Asn Leu Pro Gln Ser Gln Asn
            995   1000   1005

Ile Thr Thr Met Ile Lys Asn Ile Thr Ala Arg Asn Val Leu Gln
    1010   1015   1020

Asp Ser Pro Asn Pro Leu Leu Ser Gly Leu Phe Thr Asn Thr Met
    1025   1030   1035

Ile Glu Glu Asp Glu Glu Leu Ala Glu Phe Leu Met Asp Arg Lys
    1040   1045   1050

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Val Ile Leu Pro Arg Val Ala His Asp Ile Leu Asp Asn Ser Leu
    1055   1060   1065

Thr Gly Ile Arg Asn Ala Ile Ala Gly Met Leu Asp Thr Thr Lys
    1070   1075   1080

Ser Leu Ile Arg Val Gly Ile Asn Arg Gly Gly Leu Thr Tyr Ser
    1085   1090   1095

Leu Leu Arg Lys Ile Ser Asn Tyr Asp Leu Val Gln Tyr Glu Thr
    1100   1105   1110

Leu Ser Arg Thr Leu Arg Leu Ile Val Ser Asp Lys Ile Arg Tyr
    1115   1120   1125

Glu Asp Met Cys Ser Val Asp Leu Ala Ile Ala Leu Arg Gln Lys
    1130   1135   1140

Met Trp Ile His Leu Ser Gly Gly Arg Met Ile Ser Gly Leu Glu
    1145   1150   1155

Thr Pro Asp Pro Leu Glu Leu Leu Ser Gly Val Ile Ile Thr Gly
    1160   1165   1170

Ser Glu His Cys Lys Ile Cys Tyr Ser Ser Asp Gly Thr Asn Pro
    1175   1180   1185

Tyr Thr Trp Met Tyr Leu Pro Gly Asn Ile Lys Ile Gly Ser Ala
    1190   1195   1200

Glu Thr Gly Ile Ser Ser Leu Arg Val Pro Tyr Phe Gly Ser Val
    1205   1210   1215

Thr Asp Glu Arg Ser Glu Ala Gln Leu Gly Tyr Ile Lys Asn Leu
    1220   1225   1230

Ser Lys Pro Ala Lys Ala Ala Ile Arg Ile Ala Met Ile Tyr Thr
    1235   1240   1245

Trp Ala Phe Gly Asn Asp Glu Ile Ser Trp Met Glu Ala Ser Gln
    1250   1255   1260

Ile Ala Gln Thr Arg Ala Asn Phe Thr Leu Asp Ser Leu Lys Ile
    1265   1270   1275

```
Leu Thr Pro Val Ala Thr Ser  Thr Asn Leu Ser His  Arg Leu Lys
    1280            1285            1290

Asp Thr Ala Thr Gln Met Lys  Phe Ser Ser Thr Ser  Leu Ile Arg
    1295            1300            1305

Val Ser Arg Phe Ile Thr Met  Ser Asn Asp Asn Met  Ser Ile Lys
    1310            1315            1320

Glu Ala Asn Glu Thr Lys Asp  Thr Asn Leu Ile Tyr  Gln Gln Ile
    1325            1330            1335

Met Leu Thr Gly Leu Ser Val  Phe Glu Tyr Leu Phe  Arg Leu Glu
    1340            1345            1350

Glu Thr Thr Gly His Asn Pro  Ile Val Met His Leu  His Ile Glu
    1355            1360            1365

Asp Glu Cys Cys Ile Lys Glu  Ser Phe Asn Asp Glu  His Ile Asn
    1370            1375            1380

Pro Glu Ser Thr Leu Glu Leu  Ile Arg Tyr Pro Glu  Ser Asn Glu
    1385            1390            1395

Phe Ile Tyr Asp Lys Asp Pro  Leu Lys Asp Val Asp  Leu Ser Lys
    1400            1405            1410

Leu Met Val Ile Lys Asp His  Ser Tyr Thr Ile Asp  Met Asn Tyr
    1415            1420            1425

Trp Asp Asp Thr Asp Ile Ile  His Ala Ile Ser Ile  Cys Thr Ala
    1430            1435            1440

Ile Thr Ile Ala Asp Thr Met  Ser Gln Leu Asp Arg  Asp Asn Leu
    1445            1450            1455

Lys Glu Ile Ile Val Ile Ala  Asn Asp Asp Asp Ile  Asn Ser Leu
    1460            1465            1470

Ile Thr Glu Phe Leu Thr Leu  Asp Ile Leu Val Phe  Leu Lys Thr
    1475            1480            1485

Phe Gly Gly Leu Leu Val Asn  Gln Phe Ala Tyr Thr  Leu Tyr Ser
    1490            1495            1500
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```
Leu Lys Thr Glu Gly Arg Asp   Leu Ile Trp Asp Tyr   Ile Met Arg
    1505                1510                1515

Thr Leu Arg Asp Thr Ser His   Ser Ile Leu Lys Val   Leu Ser Asn
    1520                1525                1530

Ala Leu Ser His Pro Lys Val   Phe Lys Arg Phe Trp   Asp Cys Gly
    1535                1540                1545

Val Leu Asn Pro Ile Tyr Gly   Pro Asn Thr Ala Ser   Gln Asp Gln
    1550                1555                1560

Ile Lys Leu Ala Leu Ser Ile   Cys Glu Tyr Ser Leu   Asp Leu Phe
    1565                1570                1575

Met Arg Glu Trp Leu Asn Gly   Val Ser Leu Glu Ile   Tyr Ile Cys
    1580                1585                1590

Asp Ser Asp Met Glu Val Ala   Asn Asp Arg Lys Gln   Ala Phe Ile
    1595                1600                1605

Ser Arg His Leu Ser Phe Val   Cys Cys Leu Ala Glu   Ile Ala Ser
    1610                1615                1620

Phe Gly Pro Asn Leu Leu Asn   Leu Thr Tyr Leu Glu   Arg Leu Asp
    1625                1630                1635

Leu Leu Lys Gln Tyr Leu Glu   Leu Asn Ile Lys Asp   Asp Pro Thr
    1640                1645                1650

Leu Lys Tyr Val Gln Ile Ser   Gly Leu Leu Ile Lys   Ser Phe Pro
    1655                1660                1665

Ser Thr Val Thr Tyr Val Arg   Lys Thr Ala Ile Lys   Tyr Leu Arg
    1670                1675                1680

Ile Arg Gly Ile Ser Pro Pro   Glu Val Ile Asp Asp   Trp Asp Pro
    1685                1690                1695

Ile Glu Asp Glu Asn Met Leu   Asp Asn Ile Val Lys   Thr Ile Asn
    1700                1705                1710

Asp Asn Cys Asn Lys Asp Asn   Lys Gly Asn Lys Ile   Asn Asn Phe
    1715                1720                1725
```

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```
Trp Gly Leu Ala Leu Lys Asn   Tyr Gln Val Leu Lys   Ile Arg Ser
    1730              1735               1740


Ile Thr Ser Asp Ser Asp Asn   Asn Asp Arg Ser Asp   Ala Ser Thr
    1745              1750               1755


Gly Gly Leu Thr Leu Pro Gln   Gly Gly Asn Tyr Leu   Ser His Gln
    1760              1765               1770


Leu Arg Leu Phe Gly Ile Asn   Ser Thr Ser Cys Leu   Lys Ala Leu
    1775              1780               1785


Glu Leu Ser Gln Ile Leu Met   Lys Glu Val Asn Lys   Asp Gln Asp
    1790              1795               1800


Arg Leu Phe Leu Gly Glu Gly   Ala Gly Ala Met Leu   Ala Cys Tyr
    1805              1810               1815


Asp Ala Thr Leu Gly Pro Ala   Val Asn Tyr Tyr Asn   Ser Gly Leu
    1820              1825               1830


Asn Ile Thr Asp Val Ile Gly   Gln Arg Glu Leu Lys   Ile Phe Pro
    1835              1840               1845


Ser Glu Val Ser Leu Val Gly   Lys Lys Leu Gly Asn   Val Thr Gln
    1850              1855               1860


Ile Leu Asn Arg Val Lys Val   Leu Phe Asn Gly Asn   Pro Asn Ser
    1865              1870               1875


Thr Trp Ile Gly Asn Met Glu   Cys Glu Thr Leu Ile   Trp Ser Glu
    1880              1885               1890


Leu Asn Asp Lys Ser Ile Gly   Leu Val His Cys Asp   Met Glu Gly
    1895              1900               1905


Ala Ile Gly Lys Ser Glu Glu   Thr Val Leu His Glu   His Tyr Ser
    1910              1915               1920


Val Ile Arg Ile Thr Tyr Leu   Ile Gly Asp Asp Asp   Val Val Leu
    1925              1930               1935


Ile Ser Lys Ile Ile Pro Thr   Ile Thr Pro Asn Trp   Ser Arg Ile
    1940              1945               1950
```

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```
Leu Tyr  Leu Tyr Lys Leu Tyr  Trp Lys Asp Val Ser  Ile Ile Ser
    1955             1960             1965


Leu Lys  Thr Ser Asn Pro Ala  Ser Thr Glu Leu Tyr  Leu Ile Ser
    1970             1975             1980


Lys Asp  Ala Tyr Cys Thr Ile  Met Glu Pro Ser Glu  Val Val Leu
    1985             1990             1995


Ser Lys  Leu Lys Arg Leu Ser  Leu Leu Glu Glu Asn  Asn Leu Leu
    2000             2005             2010


Lys Trp  Ile Ile Leu Ser Lys  Lys Lys Asn Asn Glu  Trp Leu His
    2015             2020             2025


His Glu  Ile Lys Glu Gly Glu  Arg Asp Tyr Gly Val  Met Arg Pro
    2030             2035             2040


Tyr His  Met Ala Leu Gln Ile  Phe Gly Phe Gln Ile  Asn Leu Asn
    2045             2050             2055


His Leu  Ala Lys Glu Phe Leu  Ser Thr Pro Asp Leu  Thr Asn Ile
    2060             2065             2070


Asn Asn  Ile Ile Gln Ser Phe  Gln Arg Thr Ile Lys  Asp Val Leu
    2075             2080             2085


Phe Glu  Trp Ile Asn Ile Thr  His Asp Gly Lys Arg  His Lys Leu
    2090             2095             2100


Gly Gly  Arg Tyr Asn Ile Phe  Pro Leu Lys Asn Lys  Gly Lys Leu
    2105             2110             2115


Arg Leu  Leu Ser Arg Arg Leu  Val Leu Ser Trp Ile  Ser Leu Ser
    2120             2125             2130


Leu Ser  Thr Arg Leu Leu Thr  Gly Arg Phe Pro Asp  Glu Lys Phe
    2135             2140             2145


Glu His  Arg Ala Gln Thr Gly  Tyr Val Ser Leu Pro  Asp Thr Asp
    2150             2155             2160


Leu Glu  Ser Leu Lys Leu Leu  Ser Lys Asn Thr Ile  Lys Asn Tyr
    2165             2170             2175
```

67

```
Arg Glu  Cys Ile Gly Ser Ile   Ser Tyr Trp Phe Leu  Thr Lys Glu
    2180               2185               2190
```

```
Val Lys  Ile Leu Met Lys Leu   Ile Gly Gly Ala Lys  Leu Leu Gly
    2195               2200               2205
```

```
Ile Pro  Arg Gln Tyr Lys Glu   Pro Glu Glu Gln Leu  Leu Glu Asp
    2210               2215               2220
```

```
Tyr Asn  Gln His Asp Glu Phe   Asp Ile Asp
    2225               2230
```

```
<210>  15
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<212>  PRT
<213>  human parainfluenza virus 1
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```
<400>  15
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```
Met Asp Lys Gln Glu Ser Thr Gln Asn Ser Ser Asp Ile Leu Tyr Pro
1               5               10              15
```

```
Glu Cys His Leu Asn Ser Pro Ile Val Lys Ser Lys Ile Ala Gln Leu
            20              25              30
```

```
His Val Leu Leu Asp Ile Asn Gln Pro Tyr Asp Leu Lys Asp Asn Ser
        35              40              45
```

```
Ile Ile Asn Ile Thr Lys Tyr Lys Ile Arg Asn Gly Gly Leu Ser Pro
    50              55              60
```

```
Arg Gln Ile Lys Ile Arg Ser Leu Gly Lys Ile Leu Lys Gln Glu Ile
65              70              75              80
```

```
Lys Asp Ile Asp Arg Tyr Thr Phe Glu Pro Tyr Pro Ile Phe Ser Leu
                85              90              95
```

```
Glu Leu Leu Arg Leu Asp Ile Pro Glu Ile Cys Asp Lys Ile Arg Ser
            100             105             110
```

```
Ile Phe Ser Val Ser Asp Arg Leu Ile Arg Glu Leu Ser Ser Gly Phe
        115             120             125
```

```
Gln Glu Leu Trp Leu Asn Ile Leu Arg Gln Leu Gly Cys Val Glu Gly
    130             135             140
```

Lys Glu Gly Phe Asp Ser Leu Lys Asp Val Asp Ile Ile Pro Asp Ile
145   150   155   160

Thr Asp Lys Tyr Asn Lys Asn Thr Trp Tyr Arg Pro Phe Leu Thr Trp
                165   170   175

Phe Ser Ile Lys Tyr Asp Met Arg Trp Met Gln Lys Asn Lys Ser Gly
            180   185   190

Asn His Leu Asp Val Ser Asn Ser His Asn Phe Leu Asp Cys Lys Ser
        195   200   205

Tyr Ile Leu Ile Ile Tyr Arg Asp Leu Val Ile Ile Ile Asn Lys Leu
    210   215   220

Lys Leu Thr Gly Tyr Val Leu Thr Pro Glu Leu Val Leu Met Tyr Cys
225   230   235   240

Asp Val Val Glu Gly Arg Trp Asn Met Ser Ser Ala Gly Arg Leu Asp
            245   250   255

Lys Arg Ser Ser Lys Ile Thr Cys Lys Gly Glu Glu Leu Trp Glu Leu
        260   265   270

Ile Asp Ser Leu Phe Pro Asn Leu Gly Glu Asp Val Tyr Asn Ile Ile
        275   280   285

Ser Leu Leu Glu Pro Leu Ser Leu Ala Leu Ile Gln Leu Asp Asp Pro
290   ,295   300

Val Thr Asn Leu Lys Gly Ala Phe Met Arg His Val Leu Thr Glu Leu
305   310   315   320

His Thr Ile Leu Ile Lys Asp Asn Ile Tyr Thr Asp Ser Glu Ala Asp
            325   330   335

Ser Ile Met Glu Ser Leu Ile Lys Ile Phe Arg Glu Thr Ser Ile Asp
        340   345   350

Glu Lys Ala Glu Ile Phe Ser Phe Phe Arg Thr Phe Gly His Pro Ser
        355   360   365

Leu Glu Ala Ile Thr Ala Ala Asp Lys Val Arg Thr His Met Tyr Ser
370   375   380

Ser Lys Lys Ile Ile Leu Lys Thr Leu Tyr Glu Cys His Ala Ile Phe
385   390   395   400

Cys Ala Ile Ile Ile Asn Gly Tyr Arg Glu Arg His Gly Gly Gln Trp
                405   410   415

Pro Pro Cys Glu Phe Pro Asn His Val Cys Leu Glu Leu Lys Asn Ala
            420   425   430

Gln Gly Ser Asn Ser Ala Ile Ser Tyr Glu Cys Ala Val Asp Asn Tyr
            435   440   445

Ser Ser Phe Ile Gly Phe Lys Phe Leu Lys Phe Ile Glu Pro Gln Leu
            450   455   460

Asp Glu Asp Leu Thr Ile Tyr Met Lys Asp Lys Ala Leu Ser Pro Arg
465   470   475   480

Lys Ala Ala Trp Asp Ser Val Tyr Pro Asp Ser Asn Leu Tyr Tyr Lys
                485   490   495

Val Pro Glu Ser Glu Glu Thr Arg Arg Leu Ile Glu Val Phe Ile Asn
            500   505   510

Asp Asn Asn Phe Asn Pro Ala Asp Ile Ile Asn Tyr Val Glu Ser Gly
            515   520   525

Glu Trp Leu Asn Asp Asp Ser Phe Asn Ile Ser Tyr Ser Leu Lys Glu
            530   535   540

Lys Glu Ile Lys Gln Glu Gly Arg Leu Phe Ala Lys Met Thr Tyr Lys
545   550   555   560

Met Arg Ala Val Gln Val Leu Ala Glu Thr Leu Leu Ala Lys Gly Val
                565   570   575

Gly Glu Leu Phe Ser Glu Asn Gly Met Val Lys Gly Glu Ile Asp Leu
            580   585   590

Leu Lys Arg Leu Thr Thr Leu Ser Val Ser Gly Val Pro Arg Ser Asn
            595   600   605

Ser Val Tyr Asn Asn Pro Ile Leu His Glu Lys Leu Ile Lys Asn Met
610   615   620

70

Asn Lys Cys Asn Ser Asn Gly Tyr Trp Asp Glu Arg Lys Lys Ser Lys
625   630   635   640

Asn Glu Phe Lys Ala Ala Asp Ser Ser Thr Glu Gly Tyr Glu Thr Leu
            645   650   655

Ser Cys Phe Leu Thr Thr Asp Leu Lys Lys Tyr Cys Leu Asn Trp Arg
            660   665   670

Phe Glu Ser Thr Ala Leu Phe Gly Gln Arg Cys Asn Glu Ile Phe Gly
            675   680   685

Phe Lys Thr Phe Phe Asn Trp Met His Pro Ile Leu Glu Lys Ser Thr
    690   695   700

Ile Tyr Val Gly Asp Pro Tyr Cys Pro Val Pro Asp Arg Met His Lys
705   710   715   720

Glu Leu Gln Asp His Asp Asp Thr Gly Ile Phe Ile His Asn Pro Arg
            725   730   735

Gly Gly Ile Glu Gly Tyr Cys Gln Lys Leu Trp Thr Leu Ile Ser Ile
            740   745   750

Ser Ala Ile His Leu Ala Ala Val Lys Val Gly Val Arg Val Ser Ala
            755   760   765

Met Val Gln Gly Asp Asn Gln Ala Ile Ala Val Thr Ser Arg Val Pro
    770   775   780

Val Thr Gln Thr Tyr Lys Gln Lys Lys Thr His Val Tyr Glu Glu Ile
785   790   795   800

Thr Arg Tyr Phe Gly Ala Leu Arg Glu Val Met Phe Asp Ile Gly His
            805   810   815

Glu Leu Lys Leu Asn Glu Thr Ile Ile Ser Ser Lys Met Phe Val Tyr
            820   825   830

Ser Lys Arg Ile Tyr Tyr Asp Gly Lys Ile Leu Pro Gln Cys Leu Lys
            835   840   845

Ala Leu Thr Arg Cys Val Phe Trp Ser Glu Thr Leu Val Asp Glu Asn
    850   855   860

71

Arg Ser Ala Cys Ser Asn Ile Ala Thr Ser Ile Ala Lys Ala Ile Glu
865   870   875   880

Asn Gly Tyr Ser Pro Ile Leu Gly Tyr Cys Ile Ala Leu Phe Lys Thr
                885   890   895

Cys Gln Gln Val Cys Ile Ser Leu Gly Met Thr Ile Asn Pro Thr Ile
            900   905   910

Thr Ser Thr Ile Lys Asp Gln Tyr Phe Lys Gly Lys Asn Trp Leu Arg
            915   920   925

Cys Ala Ile Leu Ile Pro Ala Asn Ile Gly Gly Phe Asn Tyr Met Ser
    930   935   940

Thr Ala Arg Cys Phe Val Arg Asn Ile Gly Asp Pro Ala Val Ala Ala
945   950   955   960

Leu Ala Asp Leu Lys Arg Phe Ile Lys Ala Gly Leu Leu Asp Lys Gln
            965   970   975

Val Leu Tyr Arg Val Met Asn Gln Glu Pro Gly Asp Ser Ser Phe Leu
            980   985   990

Asp Trp Ala Ser Asp Pro Tyr Ser Cys Asn Leu Pro His Ser Gln Ser
        995   1000   1005

Ile Thr Thr Ile Ile Lys Asn Val Thr Ala Arg Ser Val Leu Gln
    1010   1015   1020

Glu Ser Pro Asn Pro Leu Leu Ser Gly Leu Phe Ser Glu Ser Ser
    1025   1030   1035

Ser Glu Glu Asp Leu Asn Leu Ala Ser Phe Leu Met Asp Arg Lys
    1040   1045   1050

Ala Ile Leu Pro Arg Val Ala His Glu Ile Leu Asp Asn Ser Leu
    1055   1060   1065

Thr Gly Val Arg Glu Ala Ile Ala Gly Met Leu Asp Thr Thr Lys
    1070   1075   1080

Ser Leu Val Arg Ala Ser Val Arg Arg Gly Gly Leu Ser Tyr Ser
    1085   1090   1095

72

```
Ile Leu Arg Arg Leu Ile Asn   Tyr Asp Leu Leu Gln   Tyr Glu Thr
    1100                1105                 1110

Leu Thr Arg Thr Leu Arg Lys   Pro Val Lys Asp Asn   Ile Glu Tyr
    1115                1120                 1125

Glu Tyr Met Cys Ser Val Glu   Leu Ala Ile Gly Leu   Arg Gln Lys
    1130                1135                 1140

Met Trp Phe His Leu Thr Tyr   Gly Arg Pro Ile His   Gly Leu Glu
    1145                1150                 1155

Thr Pro Asp Pro Leu Glu Leu   Leu Arg Gly Ser Phe   Ile Glu Gly
    1160                1165                 1170

Ser Glu Ile Cys Lys Phe Cys   Arg Ser Glu Gly Asn   Asn Pro Met
    1175                1180                 1185

Tyr Thr Trp Phe Tyr Leu Pro   Asp Asn Ile Asp Leu   Asp Thr Leu
    1190                1195                 1200

Ser Asn Gly Ser Pro Ala Ile   Arg Ile Pro Tyr Phe   Gly Ser Ala
    1205                1210                 1215

Thr Asp Glu Arg Ser Glu Ala   Gln Leu Gly Tyr Val   Lys Asn Leu
    1220                1225                 1230

Ser Lys Pro Ala Lys Ala Ala   Ile Arg Ile Ala Met   Val Tyr Thr
    1235                1240                 1245

Trp Ala Tyr Gly Thr Asp Glu   Ile Ser Trp Met Glu   Ala Ala Leu
    1250                1255                 1260

Ile Ala Gln Thr Arg Ala Asn   Leu Ser Leu Glu Asn   Leu Lys Leu
    1265                1270                 1275

Leu Thr Pro Val Ser Thr Ser   Thr Asn Leu Ser His   Arg Leu Arg
    1280                1285                 1290

Asp Thr Ala Thr Gln Met Lys   Phe Ser Ser Ala Thr   Leu Val Arg
    1295                1300                 1305

Ala Ser Arg Phe Ile Thr Ile   Ser Asn Asp Asn Met   Ala Leu Lys
    1310                1315                 1320
```

Glu Ala Gly Glu Ser Lys Asp Thr Asn Leu Val Tyr Gln Gln Ile
    1325   1330   1335

Met Leu Thr Gly Leu Ser Leu Phe Glu Phe Asn Met Arg Tyr Lys
    1340   1345   1350

Gln Gly Ser Leu Ser Lys Pro Met Ile Leu His Leu His Leu Asn
    1355   1360   1365

Asn Lys Cys Cys Ile Ile Glu Ser Pro Gln Glu Leu Asn Ile Pro
    1370   1375   1380

Pro Arg Ser Thr Leu Asp Leu Glu Ile Thr Gln Glu Asn Asn Lys
    1385   1390   1395

Leu Ile Tyr Asp Pro Asp Pro Leu Lys Asp Ile Asp Leu Glu Leu
    1400   1405   1410

Phe Ser Lys Val Arg Asp Val Val His Thr Ile Asp Met Asn Tyr
    1415   1420   1425

Trp Ser Asp Asp Glu Ile Ile Arg Ala Thr Ser Ile Cys Thr Ala
    1430   1435   1440

Met Thr Ile Ala Asp Thr Met Ser Gln Leu Asp Arg Asp Asn Leu
    1445   1450   1455

Lys Glu Met Ile Ala Leu Ile Asn Asp Asp Asp Ile Asn Ser Leu
    1460   1465   1470

Ile Thr Glu Phe Met Val Ile Asp Ile Pro Leu Phe Cys Ser Thr
    1475   1480   1485

Phe Gly Gly Ile Leu Ile Asn Gln Phe Ala Tyr Ser Leu Tyr Gly
    1490   1495   1500

Leu Asn Val Arg Gly Arg Asp Glu Ile Trp Gly Tyr Val Ile Arg
    1505   1510   1515

Ile Ile Lys Asp Thr Ser His Ala Val Leu Lys Val Leu Ser Asn
    1520   1525   1530

Ala Leu Ser His Pro Lys Ile Phe Lys Arg Phe Trp Asp Ala Gly
    1535   1540   1545

Val Val Glu Pro Val Tyr Gly Pro Asn Leu Ser Asn Gln Asp Lys
1550   1555   1560

Ile Leu Leu Ala Ile Ser Val Cys Glu Tyr Ser Val Asp Leu Phe
1565   1570   1575

Met Arg Asp Trp Gln Glu Gly Ile Pro Leu Glu Ile Phe Ile Cys
1580   1585   1590

Asp Asn Asp Pro Asn Ile Ala Glu Met Arg Lys Leu Ser Phe Leu
1595   1600   1605

Ala Arg His Leu Ala Tyr Leu Cys Ser Leu Ala Glu Ile Ala Lys
1610   1615   1620

Glu Gly Pro Lys Leu Glu Ser Met Thr Ser Leu Glu Arg Leu Glu
1625   1630   1635

Ser Leu Lys Glu Tyr Leu Glu Leu Thr Phe Leu Asp Asp Pro Ile
1640   1645   1650

Leu Arg Tyr Ser Gln Leu Thr Gly Leu Val Ile Lys Ile Phe Pro
1655   1660   1665

Ser Thr Leu Thr Tyr Ile Arg Lys Ser Ser Ile Lys Val Leu Arg
1670   1675   1680

Val Arg Gly Ile Gly Ile Pro Glu Val Leu Glu Asp Trp Asp Pro
1685   1690   1695

Asp Ala Asp Ser Met Leu Leu Asp Asn Ile Thr Ala Glu Val Gln
1700   1705   1710

His Asn Ile Pro Leu Lys Lys Asn Glu Arg Thr Pro Phe Trp Gly
1715   1720   1725

Leu Arg Val Ser Lys Ser Gln Val Leu Arg Leu Arg Gly Tyr Glu
1730   1735   1740

Glu Ile Lys Arg Glu Glu Arg Gly Arg Ser Gly Val Gly Leu Thr
1745   1750   1755

Leu Pro Phe Asp Gly Arg Tyr Leu Ser His Gln Leu Arg Leu Phe
1760   1765   1770

75

Gly Ile Asn Ser Thr Ser Cys Leu Lys Ala Leu Glu Leu Thr Tyr
    1775   1780   1785

Leu Leu Asn Pro Leu Val Asn Lys Asp Lys Asp Arg Leu Tyr Leu
    1790   1795   1800

Gly Glu Gly Ala Gly Ala Met Leu Ser Cys Tyr Asp Ala Thr Leu
    1805   1810   1815

Gly Pro Cys Met Asn Tyr Tyr Asn Ser Gly Val Asn Ser Cys Asp
    1820   1825   1830

Leu Asn Gly Gln Arg Glu Leu Asn Ile Tyr Pro Ser Glu Val Ala
    1835   1840   1845

Leu Val Gly Lys Lys Leu Asn Asn Val Thr Ser Leu Cys Gln Arg
    1850   1855   1860

Val Lys Val Leu Phe Asn Gly Asn Pro Gly Ser Thr Trp Ile Gly
    1865   1870   1875

Asn Asp Glu Cys Glu Thr Leu Ile Trp Asn Glu Leu Gln Asn Asn
    1880   1885   1890

Ser Ile Gly Phe Ile His Cys Asp Met Glu Gly Gly Glu His Lys
    1895   1900   1905

Cys Asp Gln Val Val Leu His Glu His Tyr Ser Val Ile Arg Ile
    1910   1915   1920

Ala Tyr Leu Val Gly Asp Lys Asp Val Ile Leu Val Ser Lys Ile
    1925   1930   1935

Ala Pro Arg Leu Gly Thr Asp Trp Thr Lys Gln Leu Ser Leu Tyr
    1940   1945   1950

Leu Arg Tyr Trp Arg Asp Val Ser Leu Ile Val Leu Lys Thr Ser
    1955   1960   1965

Asn Pro Ala Ser Thr Glu Met Tyr Leu Ile Ser Lys Asp Pro Lys
    1970   1975   1980

Ser Asp Ile Ile Glu Asp Ser Asn Thr Val Leu Ala Asn Leu Leu
    1985   1990   1995

76

Pro Leu Ser Lys Glu Asp Ser Ile Lys Ile Glu Lys Trp Ile Leu
    2000   2005   2010

Val Glu Lys Ala Lys Val His Asp Trp Ile Val Arg Glu Leu Lys
    2015   2020   2025

Glu Gly Ser Ala Ser Ser Gly Met Leu Arg Pro Tyr His Gln Ala
    2030   2035   2040

Leu Gln Ile Phe Gly Phe Glu Pro Asn Leu Asn Lys Leu Cys Arg
    2045   2050   2055

Asp Phe Leu Ser Thr Leu Asn Ile Val Asp Thr Lys Asn Cys Ile
    2060   2065   2070

Ile Thr Phe Asp Arg Val Leu Arg Asp Thr Ile Phe Glu Trp Thr
    2075   2080   2085

Arg Ile Lys Asp Ala Asp Lys Lys Leu Arg Leu Thr Gly Lys Tyr
    2090   2095   2100

Asp Leu Tyr Pro Leu Arg Asp Ser Gly Lys Leu Lys Val Ile Ser
    2105   2110   2115

Arg Arg Leu Val Ile Ser Trp Ile Ala Leu Ser Met Ser Thr Arg
    2120   2125   2130

Leu Val Thr Gly Ser Phe Pro Asp Ile Lys Phe Glu Ser Arg Leu
    2135   2140   2145

Gln Leu Gly Ile Val Ser Ile Ser Ser Arg Glu Ile Lys Asn Leu
    2150   2155   2160

Arg Val Ile Ser Lys Ile Val Ile Asp Lys Phe Glu Asp Ile Ile
    2165   2170   2175

His Ser Val Thr Tyr Arg Phe Leu Thr Lys Glu Ile Lys Ile Leu
    2180   2185   2190

Met Lys Ile Leu Gly Ala Val Lys Leu Phe Gly Ala Arg Gln Ser
    2195   2200   2205

Thr Ser Ala Asp Ile Thr Asn Ile Asp Thr Ser Asp Ser Ile Gln
    2210   2215   2220

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<212>   PRT
<213>   bovine parainfluenza virus 3

<400>   16
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```
Met Asp Thr Glu Ser His Ser Gly Thr Thr Ser Asp Ile Leu Tyr Pro
1               5                   10                  15


Glu Cys His Leu Asn Ser Pro Ile Val Lys Gly Lys Ile Ala Gln Leu
            20                  25                  30


His Thr Ile Met Ser Leu Pro Gln Pro Tyr Asp Met Asp Asp Asp Ser
            35                  40                  45


Ile Leu Ile Ile Thr Arg Gln Lys Ile Lys Leu Asn Lys Leu Asp Lys
        50                  55                  60


Arg Gln Arg Ser Ile Arg Lys Leu Arg Ser Val Leu Met Glu Arg Val
65                  70                  75                  80


Ser Asp Leu Gly Lys Tyr Thr Phe Ile Arg Tyr Pro Glu Met Ser Ser
                85                  90                  95


Glu Met Phe Gln Leu Cys Ile Pro Gly Ile Asn Asn Lys Ile Asn Glu
            100                 105                 110


Leu Leu Ser Lys Ala Ser Lys Thr Tyr Asn Gln Met Thr Asp Gly Leu
            115                 120                 125


Arg Asp Leu Trp Val Thr Ile Leu Ser Lys Leu Ala Ser Lys Asn Asp
        130                 135                 140


Gly Ser Asn Tyr Asp Ile Asn Glu Asp Ile Ser Asn Ile Ser Asn Val
145                 150                 155                 160


His Met Thr Tyr Gln Ser Asp Lys Trp Tyr Asn Pro Phe Lys Thr Trp
                165                 170                 175


Phe Thr Ile Lys Tyr Asp Met Arg Arg Leu Gln Lys Ala Lys Asn Glu
            180                 185                 190


Ile Thr Phe Asn Arg His Lys Asp Tyr Asn Leu Leu Glu Asp Gln Lys
            195                 200                 205
```

Asn Ile Leu Leu Ile His Pro Glu Leu Val Leu Ile Leu Asp Lys Gln
    210   215   220

Asn Tyr Asn Gly Tyr Ile Met Thr Pro Glu Leu Val Leu Met Tyr Cys
225   230   235   240

Asp Val Val Glu Gly Arg Trp Asn Ile Ser Ser Cys Ala Lys Leu Asp
                245   250   255

Pro Lys Leu Gln Ser Met Tyr Tyr Lys Gly Asn Asn Leu Trp Glu Ile
            260   265   270

Ile Asp Gly Leu Phe Ser Thr Leu Gly Glu Arg Thr Phe Asp Ile Ile
        275   280   285

Ser Leu Leu Glu Pro Leu Ala Leu Ser Leu Ile Gln Thr Tyr Asp Pro
    290   295   300

Val Lys Gln Leu Arg Gly Ala Phe Leu Asn His Val Leu Ser Glu Met
305   310   315   320

Glu Leu Ile Phe Ala Ala Glu Cys Thr Thr Glu Glu Ile Pro Asn Val
                325   330   335

Asp Tyr Ile Asp Lys Ile Leu Asp Val Phe Lys Glu Ser Thr Ile Asp
            340   345   350

Glu Ile Ala Glu Ile Phe Ser Phe Phe Arg Thr Phe Gly His Pro Pro
        355   360   365

Leu Glu Ala Ser Ile Ala Ala Glu Lys Val Arg Lys Tyr Met Tyr Thr
    370   375   380

Glu Lys Cys Leu Lys Phe Asp Thr Ile Asn Lys Cys His Ala Ile Phe
385   390   395   400

Cys Thr Ile Ile Ile Asn Gly Tyr Arg Glu Arg His Gly Gly Gln Trp
                405   410   415

Pro Pro Val Thr Leu Pro Val His Ala His Glu Phe Ile Ile Asn Ala
            420   425   430

Tyr Gly Ser Asn Ser Ala Ile Ser Tyr Glu Asn Ala Val Asp Tyr Tyr
    435   440   445

```
Lys Ser Phe Ile Gly Ile Lys Phe Asp Lys Phe Ile Glu Pro Gln Leu
    450             455         460
```

```
Asp Glu Asp Leu Thr Ile Tyr Met Lys Asp Lys Ala Leu Ser Pro Lys
465             470         475                         480
```

```
Lys Ser Asn Trp Asp Thr Val Tyr Pro Ala Ser Asn Leu Leu Tyr Arg
                485             490             495
```

```
Thr Asn Val Ser His Asp Ser Arg Arg Leu Val Glu Val Phe Ile Ala
            500             505             510
```

```
Asp Ser Lys Phe Asp Pro His Gln Val Leu Asp Tyr Val Glu Ser Gly
        515             520             525
```

```
Tyr Trp Leu Asp Asp Pro Glu Phe Asn Ile Ser Tyr Ser Leu Lys Glu
    530             535             540
```

```
Lys Glu Ile Lys Gln Glu Gly Arg Leu Phe Ala Lys Met Thr Tyr Lys
545             550             555                     560
```

```
Met Arg Ala Thr Gln Val Leu Ser Glu Thr Leu Leu Ala Asn Asn Ile
            565             570             575
```

```
Gly Lys Phe Phe Gln Glu Asn Gly Met Val Lys Gly Glu Ile Glu Leu
            580             585             590
```

```
Leu Lys Arg Leu Thr Thr Ile Ser Met Ser Gly Val Pro Arg Tyr Asn
        595             600             605
```

```
Glu Val Tyr Asn Asn Ser Lys Ser His Thr Glu Glu Leu Gln Ala Tyr
    610             615             620
```

```
Asn Ala Ile Ser Ser Ser Asn Leu Ser Ser Asn Gln Lys Ser Lys Lys
625             630             635                     640
```

```
Phe Glu Phe Lys Ser Thr Asp Ile Tyr Asn Asp Gly Tyr Glu Thr Val
            645             650             655
```

```
Ser Cys Phe Leu Thr Thr Asp Leu Lys Lys Tyr Cys Leu Asn Trp Arg
            660             665             670
```

```
Tyr Glu Ser Thr Ala Leu Phe Gly Asp Thr Cys Asn Gln Ile Phe Gly
    675             680             685
```

80

Leu Lys Glu Leu Phe Asn Trp Leu His Pro Arg Leu Glu Lys Ser Thr
    690   695   700

Ile Tyr Val Gly Asp Pro Tyr Cys Pro Pro Ser Asp Ile Glu His Leu
705   710   715   720

Pro Leu Asp Asp His Pro Asp Ser Gly Phe Tyr Val His Asn Pro Lys
                725   730   735

Gly Gly Ile Glu Gly Phe Cys Gln Lys Leu Trp Thr Leu Ile Ser Ile
                740   745   /   750

Ser Ala Ile His Leu Ala Ala Val Lys Ile Gly Val Arg Val Thr Ala
                755   760   765

Met Val Gln Gly Asp Asn Gln Ala Ile Ala Val Thr Thr Arg Val Pro
    770   775   780

Asn Asn Tyr Asp Tyr Lys Val Lys Lys Glu Ile Val Tyr Lys Asp Val
785   790   795   800

Val Arg Phe Phe Asp Ser Leu Arg Glu Val Met Asp Asp Leu Gly His
                805   810   815

Glu Leu Lys Leu Asn Glu Thr Ile Ile Ser Ser Lys Met Phe Ile Tyr
                820   825   830

Ser Lys Arg Ile Tyr Tyr Asp Gly Arg Ile Leu Pro Gln Ala Leu Lys
                835   840   845

Ala Leu Ser Arg Cys Val Phe Trp Ser Glu Thr Ile Ile Asp Glu Thr
    850   855   860

Arg Ser Ala Ser Ser Asn Leu Ala Thr Ser Phe Ala Lys Ala Ile Glu
865   870   875   880

Asn Gly Tyr Ser Pro Val Leu Gly Tyr Val Cys Ser Ile Phe Lys Asn
                885   890   895

Ile Gln Gln Leu Tyr Ile Ala Leu Gly Met Asn Ile Asn Pro Thr Ile
                900   905   910

Thr Gln Asn Ile Lys Asp Gln Tyr Phe Arg Asn Ile His Trp Met Gln
                915   920   925

81

Tyr Ala Ser Leu Ile Pro Ala Ser Val Gly Gly Phe Asn Tyr Met Ala
    930   935   940

Met Ser Arg Cys Phe Val Arg Asn Ile Gly Asp Pro Thr Val Ala Ala
945   950   955   960

Leu Ala Asp Ile Lys Arg Phe Ile Lys Ala Asn Leu Leu Asp Arg Gly
                965   970   975

Val Leu Tyr Arg Ile Met Asn Gln Glu Pro Gly Glu Ser Ser Phe Leu
            980   985   990

Asp Trp Ala Ser Asp Pro Tyr Ser Cys Asn Leu Pro Gln Ser Gln Asn
        995   1000   1005

Ile Thr Thr Met Ile Lys Asn Ile Thr Ala Arg Asn Val Leu Gln
    1010   1015   1020

Asp Ser Pro Asn Pro Leu Leu Ser Gly Leu Phe Thr Ser Thr Met
    1025   1030   1035

Ile Glu Glu Asp Glu Glu Leu Ala Glu Phe Leu Met Asp Arg Arg
    1040   1045   1050

Ile Ile Leu Pro Arg Val Ala His Asp Ile Leu Asp Asn Ser Leu
    1055   1060   1065

Thr Gly Ile Arg Asn Ala Ile Ala Gly Met Leu Asp Thr Thr Lys
    1070   1075   1080

Ser Leu Ile Arg Val Gly Ile Ser Arg Gly Gly Leu Thr Tyr Asn
    1085   1090   1095

Leu Leu Arg Lys Ile Ser Asn Tyr Asp Leu Val Gln Tyr Glu Thr
    1100   1105   1110

Leu Ser Lys Thr Leu Arg Leu Ile Val Ser Asp Lys Ile Lys Tyr
    1115   1120   1125

Glu Asp Met Cys Ser Val Asp Leu Ala Ile Ser Leu Arg Gln Lys
    1130   1135   1140

Met Trp Met His Leu Ser Gly Gly Arg Met Ile Asn Gly Leu Glu
    1145   1150   1155

82

```
Thr Pro Asp Pro Leu Glu Leu  Leu Ser Gly Val Ile  Ile Thr Gly
    1160              1165              1170

Ser Glu His Cys Arg Ile Cys  Tyr Ser Thr Glu Gly  Glu Ser Pro
    1175              1180              1185

Tyr Thr Trp Met Tyr Leu Pro  Gly Asn Leu Asn Ile  Gly Ser Ala
    1190              1195              1200

Glu Thr Gly Ile Ala Ser Leu  Arg Val Pro Tyr Phe  Gly Ser Val
    1205              1210              1215

Thr Asp Glu Arg Ser Glu Ala  Gln Leu Gly Tyr Ile  Lys Asn Leu
    1220              1225              1230

Ser Lys Pro Ala Lys Ala Ala  Ile Arg Ile Ala Met  Ile Tyr Thr
    1235              1240              1245

Trp Ala Phe Gly Asn Asp Glu  Ile Ser Trp Met Glu  Ala Ser Gln
    1250              1255              1260

Ile Ala Gln Thr Arg Ala Asn  Phe Thr Leu Asp Ser  Leu Lys Ile
    1265              1270              1275

Leu Thr Pro Val Thr Thr Ser  Thr Asn Leu Ser His · Arg Leu Lys
    1280              1285              1290

Asp Thr Ala Thr Gln Met Lys  Phe Ser Ser Thr Ser  Leu Ile Arg
    1295              1300              1305

Val Ser Arg Phe Ile Thr Ile  Ser Asn Asp Asn Met  Ser Ile Lys
    1310              1315              1320

Glu Ala Asn Glu Thr Lys Asp  Thr Asn Leu Ile Tyr  Gln Gln Val
    1325              1330              1335

Met Leu Thr Gly Leu Ser Val  Phe Glu Tyr Leu Phe  Arg Leu Glu
    1340              1345              1350

Glu Ser Thr Gly His Asn Pro  Met Val Met His Leu  His Ile Glu
    1355              1360              1365

Asp Gly Cys Cys Ile Lys Glu  Ser Tyr Asn Asp Glu  His Ile Asn
    1370              1375              1380
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83

Pro Glu Ser Thr Leu Glu Leu Ile Lys Tyr Pro Glu Ser Asn Glu
    1385   1390   1395

Phe Ile Tyr Asp Lys Asp Pro Leu Lys Asp Ile Asp Leu Ser Lys
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Leu Met Val Ile Arg Asp His Ser Tyr Thr Ile Asp Met Asn Tyr
    1415   1420   1425

Trp Asp Asp Thr Asp Ile Val His Ala Ile Ser Ile Cys Thr Ala
    1430   1435   1440

Val Thr Ile Ala Asp Thr Met Ser Gln Leu Asp Arg Asp Asn Leu
    1445   1450   1455

Lys Glu Leu Val Val Ile Ala Asn Asp Asp Asp Ile Asn Ser Leu
    1460   1465   1470

Ile Thr Glu Phe Leu Thr Leu Asp Ile Leu Val Phe Leu Lys Thr
    1475   1480   1485

Phe Gly Gly Leu Leu Val Asn Gln Phe Ala Tyr Thr Leu Tyr Gly
    1490   1495   1500

Leu Lys Ile Glu Gly Arg Asp Pro Ile Trp Asp Tyr Ile Met Arg
    1505   1510   1515

Thr Leu Lys Asp Thr Ser His Ser Val Leu Lys Val Leu Ser Asn
    1520   1525   1530

Ala Leu Ser His Pro Lys Val Phe Lys Arg Phe Trp Asp Cys Gly
    1535   1540   1545

Val Leu Asn Pro Ile Tyr Gly Pro Asn Thr Ala Ser Gln Asp Gln
    1550   1555   1560

Val Lys Leu Ala Leu Ser Ile Cys Glu Tyr Ser Leu Asp Leu Phe
    1565   1570   1575

Met Arg Glu Trp Leu Asn Gly Ala Ser Leu Glu Ile Tyr Ile Cys
    1580   1585   1590

Asp Ser Asp Met Glu Ile Ala Asn Asp Arg Arg Gln Ala Phe Leu
    1595   1600   1605

```
Ser Arg  His Leu Ala Phe Val  Cys Cys Leu Ala Glu  Ile Ala Ser
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Phe Gly  Pro Asn Leu Leu Asn  Leu Thr Tyr Leu Glu  Arg Leu Asp
    1625              1630              1635

                          .
Glu Leu  Lys Gln Tyr Leu Asp  Leu Asn Ile Lys Glu  Asp Pro Thr
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Leu Lys  Tyr Val Gln Val Ser  Gly Leu Leu Ile Lys  Ser Phe Pro
    1655              1660              1665


Ser Thr  Val Thr Tyr Val Arg  Lys Thr Ala Ile Lys  Tyr Leu Arg
    1670              1675              1680


Ile Arg  Gly Ile Asn Pro Pro  Glu Thr Ile Glu Asp  Trp Asp Pro
    1685              1690              1695


Ile Glu  Asp Glu Asn Ile Leu  Asp Asn Ile Val Lys  Thr Val Asn
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Asp Asn  Cys Ser Asp Asn Gln  Lys Arg Asn Lys Ser  Ser Tyr Phe
    1715              1720              1725
                                            '

Trp Gly  Leu Ala Leu Lys Asn  Tyr Gln Val Val Lys  Ile Arg Ser
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Ile Thr  Ser Asp Ser Glu Val  Asn Glu Ala Ser Asn  Val Thr Thr
    1745              1750              1755


His Gly  Met Thr Leu Pro Gln  Gly Gly Ser Tyr Leu  Ser His Gln
    1760              1765              1770


Leu Arg  Leu Phe Gly Val Asn  Ser Thr Ser Cys Leu  Lys Ala Leu
    1775              1780              1785


Glu Leu  Ser Gln Ile Leu Met  Arg Glu Val Lys Lys  Asp Lys Asp
    1790              1795              1800


Arg Leu  Phe Leu Gly Glu Gly  Ala Gly Ala Met Leu  Ala Cys Tyr
    1805              1810              1815


Asp Ala  Thr Leu Gly Pro Ala  Ile Asn Tyr Tyr Asn  Ser Gly Leu
    1820              1825              1830
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85

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Asn Ile  Thr Asp Val Ile Gly  Gln Arg Glu Leu Lys  Ile Phe Pro
    1835                 1840                 1845


Ser Glu  Val Ser Leu Val Gly  Lys Lys Leu Gly Asn  Val Thr Gln
    1850                 1855                 1860


Ile Leu  Asn Arg Val Arg Val  Leu Phe Asn Gly Asn  Pro Asn Ser
    1865                 1870                 1875


Thr Trp  Ile Gly Asn Met Glu  Cys Glu Ser Leu Ile  Trp Ser Glu
    1880                 1885                 1890


Leu Asn  Asp Lys Ser Ile Gly  Leu Val His Cys Asp  Met Glu Gly
    1895                 1900                 1905


Ala Ile  Gly Lys Ser Glu Glu  Thr Val Leu His Glu  His Tyr Ser
    1910                 1915                 1920


Ile Ile  Arg Ile Thr Tyr Leu  Ile Gly Asp Asp Asp  Val Val Leu
    1925                 1930                 1935


Val Ser  Lys Ile Ile Pro Thr  Ile Thr Pro Asn Trp  Ser Lys Ile
    1940                 1945                 1950


Leu Tyr  Leu Tyr Lys Leu Tyr  Trp Lys Asp Val Ser  Val Val Ser
    1955                 1960                 1965


Leu Lys  Thr Ser Asn Pro Ala  Ser Thr Glu Leu Tyr  Leu Ile Ser
    1970                 1975                 1980


Lys Asp  Ala Tyr Cys Thr Val  Met Glu Pro Ser Asn  Leu Val Leu
    1985                 1990                 1995


Ser Lys  Leu Lys Arg Ile Ser  Ser Ile Glu Glu Asn  Asn Leu Leu
    2000                 2005                 2010


Lys Trp  Ile Ile Leu Ser Lys  Arg Lys Asn Asn Glu  Trp Leu Gln
    2015                 2020                 2025


His Glu  Ile Lys Glu Gly Glu  Arg Asp Tyr Gly Ile  Met Arg Pro
    2030                 2035                 2040


Tyr His  Thr Ala Leu Gln Ile  Phe Gly Phe Gln Ile  Asn Leu Asn
    2045                 2050                 2055
```

```
His Leu  Ala Arg Glu Phe Leu  Ser Thr Pro Asp Leu  Thr Asn Ile
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```
Asn Asn  Ile Ile Gln Ser Phe  Thr Arg Thr Ile Lys  Asp Val Met
    2075                 2080             2085
```

```
Phe Glu  Trp Val Asn Ile Thr  His Asp Asn Lys Arg  His Lys Leu
    2090                 2095             2100
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Gly Gly  Arg Tyr Asn Leu Phe  Pro Leu Lys Asn Lys  Gly Lys Leu
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```
Leu Ser  Thr Arg Leu Leu Thr  Gly Arg Phe Pro Asp  Glu Lys Phe
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```

```
Glu Asn  Arg Ala Gln Thr Gly  Tyr Val Ser Leu Ala  Asp Ile Asp
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```

```
Leu Glu  Ser Leu Lys Leu Leu  Ser Arg Asn Ile Val  Lys Asn Tyr
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```

```
Lys Glu  His Ile Gly Leu Ile  Ser Tyr Trp Phe Leu  Thr Lys Glu
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```

```
Val Lys  Ile Leu Met Lys Leu  Ile Gly Gly Val Lys  Leu Leu Gly
    2195                 2200             2205
```

```
Ile Pro  Lys Gln Tyr Lys Glu  Leu Glu Asp Arg Ser  Ser Gln Gly
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```
Tyr Glu  Tyr Asp Asn Glu Phe  Asp Ile Asp
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94

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97

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